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Authors: Thomas P. Roland, Matthew J. Amesbury, David M. Wilkinson, Dan J. Charman, Peter Convey, Dominic A. Hodgson, Jessica Royles, Steffen Clauß, Eckhard Völcker

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ORIGINAL PAPER

Taxonomic Implications of Morphological Complexity Within the Testate Amoeba Genus *Corythion* from the Antarctic Peninsula

Thomas P. Roland^{a,1}, Matthew J. Amesbury^a, David M. Wilkinson^b, Dan J. Charman^a, Peter Convey^c, Dominic A. Hodgson^d, Jessica Royles^{c,d}, Steffen Clauß^e, and Eckhard Völcker^e

^aGeography, College of Life and Environmental Sciences, University of Exeter, UK

^bSchool of Life Sciences, University of Lincoln, UK

^cBritish Antarctic Survey, Cambridge, UK

^dDepartment of Plant Sciences, University of Cambridge, UK

^ePenard Laboratory, Berlin, Germany

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¹Corresponding author; fax +441392 723 342

e-mail t.p.roland@exeter.ac.uk (T.P. Roland).

Precise and sufficiently detailed morphological taxonomy is vital in biology, for example in the accurate interpretation of ecological and palaeoecological datasets, especially in polar regions, where biodiversity is poor. Testate amoebae on the Antarctic Peninsula (AP) are well-documented and variations in their population size have recently been interpreted as a proxy for microbial productivity changes in response to recent regional climate change. AP testate amoeba assemblages are dominated by a small number of globally ubiquitous taxa. We examine morphological variation in *Corythion* spp. across the AP, finding clear evidence supporting the presence of two morphospecies. *Corythion constricta* (Certes 1889) was identified on the AP for the first time and has potentially been previously misidentified. Furthermore, a southerly trend of decreasing average test size in *Corythion dubium* (Taránek 1881) along the AP suggests adaptive polymorphism, although the precise drivers of this remain unclear, with analysis hindered by limited environmental data. Further work into

morphological variation in *Corythion* is needed elsewhere, alongside molecular analyses, to evaluate the potential for (pseudo)cryptic diversity within the genus. We advocate a parsimonious taxonomical approach that recognises genetic diversity but also examines and develops accurate morphological divisions and descriptions suitable for light microscopy-based ecological and palaeoecological studies.

Key words: Testate amoebae; taxonomy; Antarctica; palaeoecology; morphospecies; biogeography.

Introduction

‘The most impressive aspect of the living world is its diversity’. So wrote Ernst Mayr (1997, p. 124) 20 years ago, using this observation to introduce a discussion of the history and practice of taxonomy. He argued that, over the last 300 years, since European knowledge of biodiversity greatly expanded due to the great Enlightenment voyages of ‘discovery’, taxonomy has had to make sense of an ever-increasing range of diversity. First the new floras and faunas from around the world were described, then an increasing knowledge of aquatic organisms, and later the astonishing range of microbial diversity (e.g. Margulis and Chapman 2009); something we are only recently starting to fully comprehend via the application of modern molecular biological approaches. As part of this attempt to understand microbial diversity, within the last few decades there has been a long-running debate as to the extent to which microbial taxa exhibit cosmopolitan distributions; the so called ‘everything is everywhere’ or ‘global ubiquity’ hypothesis (Baas-Becking 1934; Finlay 2002; Fontaneto 2011). A key contribution to this debate will be clarifying the extent to which apparently cosmopolitan morphospecies may contain multiple different (pseudo)cryptic species – as Bass and Boenigk (2011, p. 101) emphasised, ‘for protists the species rank is usually much broader and less well defined, thereby contributing to the perception of broad distribution’.

Interest in testate amoebae has grown considerably in the last two decades and is reflected in an increasing number of scientific publications. However, studies concerning testate amoeba taxonomy, whether based on morphometrics or molecular phylogeny, have not increased at the same rate and the application of testate amoeba in both traditional (palaeoecology, environmental monitoring) and novel (forensics and ecotoxicology) contexts is hampered by poor taxonomy and inconsistent nomenclature, potentially leading to misinterpretations or inaccurate conclusions (Kosakyan et al. 2016a).

As with all biological groups, an accurate and sufficiently detailed morphological taxonomy is critical in studies of testate amoeba ecology and palaeoecology, as considerable differences in the environmental preferences of morphotypes within single polymorphic populations and species complexes have been observed (e.g. Bobrov et al. 1995; Singer et al. 2015), potentially limiting ecological interpretation. This is especially pertinent in areas such as the Antarctic Peninsula (AP), where overall species diversity is low

(Royles et al. 2016), potentially limiting the extent to which assemblage changes over time can be used as biological indicators of climate and/or environmental change.

Morphospecific definition in testate amoebae relies principally on variations in test composition, morphology and size, with morphological taxonomies used in palaeoecological studies necessarily based on the examination of extant amoeba in modern surface samples. Subjective judgements (and, inevitably, disagreements!) as to what level of intraspecific variation or polymorphism is acceptable are subsequently common (Charman 1999). Comprehensive biometric datasets are rare in testate amoeba morphological taxonomy and new species and morphotypes are often described based on a small number of individuals (Charman 1999), especially in regions where overall populations are low (e.g. *Argyria antarctica*, Grospietsch 1971). However, the use of minor variations in test morphometry and appearance for morphotypic delineation is complicated by the occurrence of adaptive polymorphism in certain species, such as *Trinema complanatum*, and between *Euglypha laevis* and *E. rotunda* (Schönborn 1992), or where morphological plasticity exists (e.g. Bobrov and Mazei 2004; Medioli et al. 1987; Mulot et al. 2017; Wanner 1995, 1999). Difficulties associated with species delineation based on morphological variation have also been documented in other protozoan groups (e.g. Finlay et al. 1996).

The phylogeny of testate amoebae remains highly complex, with several genera recently shown to be polyphyletic (Gomaa et al. 2012; Kosakyan et al. 2012, 2016a; Lara et al. 2008; Oliverio et al. 2014). Several recent studies also provide molecular evidence for cryptic (e.g. Kosakyan et al. 2012; Oliverio et al. 2014) and pseudo-cryptic diversity (e.g. Singer et al. 2015). Taxonomic studies based on DNA (or RNA) analysis are invaluable when examining the relationship between phenotypic and genotypic diversity in modern faunas, but possess limited applicability in (sub)fossil studies where pragmatic taxonomic schemes are still essential to undertake routine diversity and abundance assessments (Charman 1999). Morphological analysis remains the only practical method of identifying testate amoebae in palaeoecological studies (Mitchell et al. 2008). Therefore, a parsimonious approach that recognises diversity but applies realistic morphological divisions suitable for differentiation via light microscopy is often required both in (non-molecular) microbial ecology and palaeoecology.

Research into morphospecific and cryptic taxonomy within testate amoeba taxonomic complexes has been largely limited to certain genera. The Hyalospheniidae, which includes the genera *Nebela* and *Hyalosphenia*, has been the focus of detailed, often molecular, taxonomic research (Kosakyan et al. 2013, 2016a; Oliverio et al. 2014; Singer et al. 2015), with many taxa subsequently reclassified to other genera within the family (e.g. *Planocarina carinata*, *Longinebela tubulosa*, *Gibbocarina galeata* and *Cornutheca ansata* all moved from *Nebela*, Kosakyan et al. 2016b). Members of Hyalospheniidae tend to exhibit more regionally restricted distributions than many other testate amoeba families, challenging the ‘everything is everywhere’ paradigm thought to apply to testate amoebae (Heger et al. 2011; Smith and Wilkinson 2007; Smith et al. 2008), although see Finlay et al. (2004) for a contrary view of *Apodera* (*Nebela*) *vas*. The potential for variability within more ubiquitous, and apparently cosmopolitan, taxa, is currently underexplored.

The globally distributed genus *Corythion* (Euglyphida, Trinematidae; Table A.1) is both typified and dominated by the cosmopolitan type taxon *Corythion dubium* and is therefore of wide interest. *Corythion dubium* has been recorded across Europe (Amesbury et al. 2016), North America (Gehrels 2006), South America (Fernández et al. 2015), China (Li et al. 2015), Japan (Bobrov et al. 2012) and New Zealand (Charman

1997), throughout the Arctic (Bobrov and Wetterich 2012) and Antarctic (Smith 1996), as well as on a number of remote oceanic islands (e.g. Ascension Island, Wilkinson and Smith 2006; Amsterdam Island, Heger et al. 2009). Whilst *C. dubium* is absent in a small number of studies (e.g. Fernández 2015; Rhoden and Pitoni 1999), it is not possible to suggest a pattern in this absence and, therefore, a global distribution must be assumed. Despite this ubiquity, no systematic examination of the possibility of morphological plasticity or cryptic diversity within *C. dubium*, or *Corythion* more broadly, exists.

Corythion dubium is the most abundant and prevalent taxon in contemporary testate amoeba assemblages found in the moss bank deposits which form in ice-free areas across the AP and South Shetland Islands (Royles and Griffiths 2015; Royles et al. 2016) and is often dominant in other samples from the sub- and maritime Antarctic regions including those from South Georgia (Beyens et al. 1995; Smith 1982; Vincke et al. 2006), Signy Island (Smith 1973a, b, 1985), the Crozet archipelago (Vincke et al. 2004a, b, c), Amsterdam Island (Heger et al. 2009) and Marion Island (Grospietsch 1971), as well as continental Antarctica (Smith 1992). The taxon is also frequently recorded further north in Tierra del Fuego (Van Bellen et al. 2014) and the Islas Malvinas/Falkland Islands (Smith 1978). Whilst considerable size variation in this taxon has been noted in other parts of the world (Cash et al. 1915; Ogden and Hedley 1980), the dominance of *C. dubium* on the AP serves to draw greater attention to morphological differences (Royles et al. 2016) and preliminary attempts have been made to delineate between morphotypes in subfossil analyses in this region (Royles et al. 2013).

The current study represents the first assessment of morphological variation exhibited in *Corythion* populations. We present morphometric data for 2750 *Corythion* spp. tests to statistically determine systematic size variability across the AP. We report *C. constricta* for the first time on the AP and propose new criteria for its differentiation from *C. dubium* based on test length and other morphological characteristics that can be practically applied in ecological and palaeoecological studies using light microscopy. We observe a southerly trend of decreasing average test size in *Corythion dubium*, suggestive of adaptive polymorphism and discuss this in terms of protist biogeography. As a result, we identify a need for further research into the phylogeny, taxonomy and biogeography of testate amoeba taxa, like *C. dubium*, that are currently considered globally ubiquitous.

Results

A total of 2750 *Corythion* spp. individuals were analysed from 61 samples across a 600 km, six location transect over the western AP (Fig. 1). Visually, a broad north-south trend of decreasing average test size (test length vs. test width) is evident and emphasised particularly through the southern end of the transect (i.e. Ardley through to Green Islands) (Table 1, Figs 2, 3), but this directional trend is not apparent in aperture width or height (Fig. 3). In addition, at the southernmost locations (Norsel Point and Green Island), there is a noticeable division between the bulk of the individuals, as they decrease in size, and a smaller but defined group of larger specimens (Fig. 2). The overall size range of specimens found at Norsel Point and Green Island is comparable to the more northern sites where continuous size distributions exist (Table 1).

Systematic model-based cluster analysis (mclust, Supplementary Material Fig. A.1) identified four clusters within the data (Fig. 4). Visual inspection revealed that the clusters are best differentiated based on test length (Fig. 4A-C), with considerably more overlap between clusters when this dimension is excluded (Fig. 4D-F). Cluster 1 follows a noticeably tangential plane to the broadly linear plane of clusters 2, 3 and 4, suggesting that it shares less similarity with the latter clusters than they do with each other. The uncertainty in converting individuals to the classifications identified by the model is also noticeably lower when expressed using test length (Fig. 4G-L). Again, conversion uncertainty is notably lower for cluster 1 in these instances.

The first two axes of principal components analysis (PCA) explained a combined 85.478% of the variance (Fig. 5). Axis 1 (69.129%) and axis 2 (16.349%) are most closely associated with vectors representing test length and aperture width, and test breadth and aperture height, respectively. The data are largely distributed along axis one, with cluster 1 following a similar tangential plane to that described in Fig. 4, with clusters 2, 3 and 4, again, following a broadly linear plane, further supporting differentiation between these two main groups.

Clusters 1 and 2 can be effectively delineated using a 45 μm division on the axis of test length (Fig. 6) and simple linear regression of the remaining clusters 2, 3 and 4 revealed a strong positive correlation (slope = 0.69, $r^2 = 0.50$) between test length and breadth. This supports the differentiation of two morphotypes (potentially morphospecies) with the *Corythion* spp. complex present on the AP: a relatively uniform group of larger (> 45 μm) individuals, and a group of smaller (< 45 μm) individuals displaying a degree of morphometric plasticity along a length-breadth gradient. Table 2 provides a summary of the morphometric characteristics of each cluster.

Observations made based on light and scanning electron microscopy (SEM) support the existence of multiple morphotypes within the *Corythion* spp. population on the AP. Figure 7 shows SEM images of individuals from the two main groups (cluster 1, > 45 μm ; clusters 2, 3 and 4, < 45 μm) demonstrating that a number of subtle morphological characteristics also vary between the two groups. The larger individuals (> 45 μm) consistently possess a more pronounced upper pre-apertural rim of width several plates thick, rather than the single plate border seen on smaller (< 45 μm) individuals. Figure 8 shows an individual typical of cluster 1 viewed using light microscopy, highlighting the pre-apertural rim. Individuals of the larger cluster are also more elongated in their proportions, with the aperture protruding more from the main body of the test with increased size (Fig. 7D) and a test length/breadth ratio of c. 1.61 compared to c. 1.42 for the smaller group. The smaller group encompasses clusters 2, 3 and 4, with individuals ranging in length from c. 20 to 45 μm . Towards the smaller end of this range tests tend to have a less pronounced aperture (Fig. 7B) than their larger equivalents (Fig. 7C), but these differences are subtle and often inconsistent. The shape and orientation of the silica plates present on the test surface, often used to aid taxonomic identification within the order Euglyphida (Charman et al. 2000), did not vary between the two main groups. It is therefore difficult to objectively and consistently split this group further using only light microscopy, despite the identification of the three clusters within it.

By applying the cluster classifications to the morphometric data on a site-by-site basis, the southward reduction in size is emphasised (Fig. 9). Within clusters 2, 3 and 4 there is a clear southward shift towards a dominance of smaller individuals, particularly from Ardley through to Green Islands. Whilst no such systematic reduction in test size is visible in cluster 1, these individuals have strong presence in the three northernmost

sites (Signy, Elephant and Ardley Islands), but are all but absent at Barrientos Island and are present, but in greatly reduced numbers, at Norsel Point and Green Island.

Discussion

Morphological Taxonomy of *Corythion* spp. on the Antarctic Peninsula

Corythion spp. are among the most commonly and widely reported taxa in Antarctica and the sub-Antarctic islands (Smith 1992, 1996). These assemblages are typically dominated by *C. dubium*, but a single study reports the presence of other *Corythion* taxa, including several varietal forms of *C. dubium*, from a range of terrestrial and aquatic habitats in eastern Antarctica (Sudzuki 1979; see Supplementary Material Table A.1). *C. dubium* remains the only member of the genus currently reported from the AP region.

The larger size and pronounced pre-apertural rim exhibited in cluster 1 (Figs 7A, 8) are consistent with the morphological description of *Corythion constricta* (Certes 1889) as provided by Jung (1942). The tangential size distribution of cluster 1, when compared with clusters 2, 3 and 4 (Fig. 6), also supports the taxonomic validity of this cluster's identification as a separate taxon. This therefore represents the first report of *C. constricta* on the AP.

Corythion constricta was first described in southern Chile as *Trinema constricta* (Certes 1889), before being transferred to *Corythion* (Jung 1942). It has previously been suggested that *C. constricta* is endemic to southern South America (Fernández et al. 2015). The identification we report here, together with its recorded presence in eastern Antarctica (Sudzuki 1979), suggests endemism is not the case, but does not rule out a regionally restricted distribution, confined to southern South America and Antarctica. However, further reports have been made in Australia (Meisterfeld and Tan 1998), New Zealand (Hazell, 2004; Roland, T.P. unpublished) and the Netherlands (Siemensma, F.J., unpublished, www.arcella.nl) suggesting that the taxon may have a broader distribution than previously thought. Most likely owing to their comparative rarity, many members of the genus, other than *C. dubium*, are not described in commonly-used taxonomic keys (e.g. Charman et al. 2000), potentially compounding a relative absence of *C. constricta*, and other species, from contemporary and sub-fossil samples.

Cluster analysis performed within this study suggests *C. constricta* (cluster 1) can be delineated from the remaining clusters (2-4) using a critical test length of 45 µm. The largest example found in this study measured c. 61 µm. A critical test length of 55 µm was suggested by Hazell (2004) to delineate between *C. dubium* and tests matching the description of *C. constricta* found in New Zealand, and the size range for *C. constricta* found in the Netherlands is comparable to that of this study (length 49 – 72 µm; Siemensma, F.J., unpublished, www.arcella.nl). Previous studies from southern Chile (Jung 1942) and the Islas Malvinas/Falkland Islands (Vucetich 1975) provide larger size ranges for *C. constricta* than that presented in this study, of length 68-74 x breadth 50-56 µm, and length 79-83 x breadth 57-62 µm, respectively.

Whilst it is possible that specimens reported in each study may represent two, or more, distinct species, this is unlikely given the similarity in the descriptions, drawings and photographs provided. Notably, none of the studies listed here contain systematic morphometric analysis, such as that presented in this study. It is therefore clear that there is considerable potential for size variation within *C. constricta*, which has not yet been fully explored and requires further study.

The *C. constricta* tests observed on the AP were ovoid and flattened with oval plate-like idiosomes, irregularly arranged and frequently overlapping. The tests were colourless and usually transparent. The aperture was sub-terminal, oval and invaginated, and is surrounded by a series of apertural plates with median dorsal teeth. It was differentiated from *C. dubium* by the characteristically pronounced pre-apertural rim, consisting of 2 to 3 irregularly arranged plates, compared to the single, more uniformly arranged row of plates that characterised the more subtle pre-apertural rim of *C. dubium*. SEM (Fig. 7) and light microscope (Fig. 8) images of both taxa are provided. The size ranges associated with the *C. constricta* tests found on the AP are outlined in Table 1 (Cluster 1), but should not be considered exhaustive for the taxa, owing to the considerable size ranges reported in previous studies.

The great degree of size variability exhibited by *C. dubium* has long been acknowledged (Cash et al. 1915). Reported size ranges vary accordingly (e.g. length 23-65 x breadth 16-40 μm , Cash et al. 1915; length 33-55 x 24-33 μm , Ogden and Hedley 1980; length 33-43 x c. 25 μm , Siemensma, F.J., unpublished, www.arcella.nl) and are often based on a small number of individuals (e.g. $n = 3$, Ogden and Hedley 1980). Although there is some overlap between these and the ranges proposed for *C. constricta* the two taxa remain distinguishable by the presence of the pronounced pre-apertural rim, regardless of size. Further work is clearly required to fully explore the morphometric detail of members of the *Corythion* genus.

The SEM images (Fig. 7) identify subtle morphological differences across the size distribution encompassed by clusters 2-4, which could be considered partially consistent with other members of *Corythion*. For example, the smallest cluster (cluster 4) possesses some similarities in terms of overall size and circular aperture morphology with *Corythion delamarei* (Bonnet and Thomas 1960), whereas the larger cluster's (cluster 2) tendency for greater length and width, semi-circular aperture and a broader sub-circular shape is similar to *Corythion aerophila* (Decloitre 1950). The test length/breadth ratio becomes gradually lower through from cluster 4 (c. 1.48), cluster 3 (c. 1.41), to cluster 2 (c. 1.33) supporting this pattern of increasing roundness. Whilst *C. delamarei* and *C. aerophila* have both been reported in eastern Antarctica, alongside *C. dubium* (Sudzuki 1979), the rather vague original descriptions (Bonnet and Thomas 1960; Decloitre 1950) and inconsistencies in the occurrence of these features, together with the continuous and broadly linear relationship between test length and width across the size range associated with clusters 2-4, suggests that these clusters represent morphological plasticity within a single *C. dubium* classification, rather than either *C. delamarei* or *C. aerophila*, potentially drawing into question the taxonomic validity of some of these less frequently reported *Corythion* taxa.

Overall, the genus *Corythion* is taxonomically complex and often ambiguous owing to a lack of clarity and availability of the individual taxon descriptions, with at least six varietal descriptions of *C. dubium* alone (see Supplementary Material Table A.1). A detailed molecular biological study of the genus is required to elucidate the taxonomy and thereby clarify and explore ecological and biogeographical questions posed by the current morphological taxonomy. However, the new delineation of *C. constricta* in our data is clear and, in

future studies, analysts should define this taxa for *Corythion* spp. individuals > 45 µm in length and that display a thick pre-apertural margin (Figs 7, 8).

Biogeography of *Corythion* spp. on the Antarctic Peninsula

The biogeographic influence of latitude on the body size (Blackburn et al. 1999) and diversity (Mittelbach et al. 2007) of large organisms is well documented, but whether these same processes influence microbial populations is not confirmed (Fernández et al. 2016; Fontaneto 2011; Martiny et al. 2006).

Whilst the distribution and ecology of testate amoebae in Antarctica are relatively well documented (Smith 1992, 1996), assemblages typically possess lower species diversity than in temperate and tropical regions and are dominated by small, cosmopolitan taxa such as members of the genera *Corythion*, *Trinema* and *Euglypha* (Mieczan and Adamczuk 2014; Smith 1992; Smith and Wilkinson 2007; Wilkinson 2001; Yang et al. 2010). These characteristics are consistent with other cold climate environments in the sub-Antarctic (Vincke et al. 2004b, 2006) and Arctic (Beyens et al. 1986, 1990, 1992).

A broad trend of decreasing diversity, or depauperisation, in testate amoebae communities towards higher latitudes has been reported in Antarctica (Smith 1996). Testate amoebae are abundant in AP moss banks but the relative homogeneity of community structure and associated environmental gradients across the region means that uncertainty remains over the key drivers of population changes over time and space (Royles et al. 2016). Previous research suggests that temperature, moisture, pH and biogeographic factors all influence the contemporary distribution and population dynamics of moss-dwelling testate amoebae in Antarctica (Mieczan and Adamczuk 2014; Smith 1992, 1996; Todorov and Golemansky 1999; Yang et al. 2010) and the concentration, estimated biomass and species diversity of testate amoebae have been recently used as a proxy for microbial productivity in AP moss banks (Amesbury et al. 2017; Royles et al. 2013, 2016). However, in a study of testate amoeba assemblages from moss banks across the AP, no clear patterns in the species or relative abundance of taxa were discernible, leading to the suggestion, supported by microclimatic data, that living conditions for testate amoebae are relatively homogenous in the region (Royles et al. 2016). Recent summer temperature records from Orcadas (near Signy Island) and Rothera (between Green Island and Lazarev Bay) (Figure 1) are similar, despite c. 800 km of latitudinal difference, and diurnal temperature variation at the moss surface also exceeds the latitudinal AP temperature cline (Royles et al. 2016). Despite this, testate amoeba concentration, estimated biomass and species biodiversity were found to broadly decrease with latitude across this gradient from Signy Island to Lazarev Bay, although Green Island provided an exception, with samples possessing higher concentration, estimated biomass and species diversity than those from the more northerly Ardley Island or Norsel Point (Royles et al. 2016). Green Island also possesses an exceptionally rich vegetation and a suite of invertebrate fauna that are comparatively diverse for the region (Usher and Edwards 1986). This evidence suggests that variables influential in driving testate amoebae productivity in this region are complex and non-linear in their influence (Mieczan and Adamczuk 2014; Smith 1996).

The broad reduction in *C. dubium* (clusters 2-4) test size seen across the AP (Fig. 9) could be interpreted as a physiological response and/or the result of selective distribution, related to perceived harsher

climatic conditions at higher latitudes, relating to a southward reduction in growing season length. Currently, no study has been undertaken of the relationship between latitude and intra-taxon variation in test morphology.

Ecomorphosis in testate amoebae occurs when environmental or ecological factors cause variation in test morphology and can potentially be problematic taxonomically as differentiation of closely related taxa is primarily based on these characteristics, particularly in palaeoecological studies (Wanner 1999). The adaptive strategies of testate amoebae are complex and diverse, with taxon-specific mechanisms evident (Bobrov and Mazei 2004) and a range of factors capable of causing variability in test morphology including environmental variables such as temperature, food supply and trophic level (Chardez 1989; Wanner 1999; Wanner and Meisterfeld 1994).

Discussion of the mechanistic relationships between testate amoeba functional traits and environmental variables is increasingly prominent in the literature (Fournier et al. 2012, 2015; van Bellen et al. 2017) and functional diversity in testate amoebae has been linked with a set of complex interactions between environmental variables, environmental productivity and food resources (Arrieira et al. 2015). It would be reasonable to consider adaptive phenotypic plasticity, or adaptive polymorphism (cf. Schönborn 1992), as a similarly complex response to these same variables. For example, if smaller species, or those with more compressed tests, tend to be more abundant under low moisture conditions owing to their ability to thrive in thin water films (Fournier et al. 2012), it would be logical to link a reduction in the average test size of a given species, in this case *C. dubium*, to decreasing moisture availability. However, a linear response of this type is unlikely. For *Trinema enchelys*, a close relative of *C. dubium*, test size was found to be positively correlated with soil moisture but, as food consumption also varied with soil moisture, a direct response was difficult to confirm (Laminger 1978). Adding further complication, morphometric variability has been recorded in stable condition clonal cultures of *Trinema lineare*, a taxon again closely related to *C. dubium* (Schönborn 1992), indicating the presence of extensive phenotypic spectra in natural populations (Bobrov and Mazei 2004).

The AP moss banks from which the data in this study were collected become increasingly moisture-limited at higher latitudes, with *Chorisodontium aciphyllum* the dominant bank-forming species in northern sites and *Polytrichum strictum* dominating in the drier, southerly sites (Fenton and Smith 1982). No relationship could be found between the type or relative abundance of testate amoebae taxa and moss type across the AP (Royles et al. 2016) and, although *C. aciphyllum*-dominated samples appeared on average to possess marginally larger *C. dubium* individuals in the four morphometric variables than *P. strictum*-dominated samples (Figure 10), the difference was very small and the overall size ranges associated with both moss types were comparable. Furthermore, samples which were co-dominated by both moss species appear to have slightly larger *C. dubium* individuals than those dominated by one or other moss (Figure 10), suggesting that moss species, potentially as a function of moisture availability, is not a driver in determining *C. dubium* test size. PCA performed on the morphometric dataset also demonstrated almost complete overlap of 95% ellipses associated with each moss type (Supplementary Material Fig. A.2).

Understanding of precipitation patterns across the AP is limited, but model-derived predictions suggest a broadly unimodal distribution pattern of decreasing precipitation away from the central part of the western AP, with the highest precipitation experienced around the Vernadsky/Faraday research station (Fig. 1) (Turner et al. 1998). The sensitivity of Cercozoan testate amoebae, such as *Corythion*, to drought is well documented (Harder et al. 2016) but as the gradient of decreasing *C. dubium* test size is unidirectional (Fig. 9), there

appears to be no clear or direct relationship between precipitation amount and test size in this instance. It is possible that lower temperatures associated with higher latitudes, however subtle, could limit the growth of testate amoebae and their food sources (Torigai et al. 2000), potentially resulting in a southward reduction in biomass compared to individuals from lower latitudes (Ju et al. 2014). However, as temperature and moisture availability vary only slightly between sites, an alternative explanation must be sought for the southward decrease in the average test size in *C. dubium* populations on the AP. It seems likely that at high latitudes the availability of ambient energy is often as limiting as moisture or temperature alone for testate amoebae survival, growth and reproduction (Fernández et al. 2016). In this instance, a southerly decrease in ambient energy towards higher latitudes could feasibly lead to an adaptive reduction in size by *C. dubium*.

Alternatively, rather than *C. dubium* individuals adapting to less favourable environmental conditions via a morphoplastic reduction in test size, it is possible that this southerly trend is the result of smaller individuals proliferating at the expense of their larger equivalents. Smaller testate amoebae are more likely to possess cosmopolitan distributions than larger taxa, owing to their typically greater population sizes and increased propensity for wind-blown dispersal (Wilkinson 2001; Wilkinson et al. 2012). However, it seems unlikely that a mechanism of this order could be responsible for the broad decrease in *C. dubium* test size southward across the AP, especially as the larger *C. constricta* (cluster 1) is present at the two most southerly sites in the transect. The apparent absence of *C. constricta* from Barrientos Island could be a product of the moss deposits found here being shallower and drier, owing to a more free-draining, sandy substrate, and therefore representing a slightly different environment to the other sites on the transect.

Small generalist species, such as *Corythion* spp., are considered early colonizers of new habitats (Fournier et al. 2016; Lara et al. 2011; Smith et al. 2008) and recently deglaciated areas (Carlson et al. 2010; Fernández et al. 2012; Wilkinson 1990). It is possible that the southward trend of decreasing average test size is the result of preferential colonisation of recently formed moss banks by smaller *C. dubium* individuals, in keeping with the regional north to south pattern of deglaciation (Ó Cofaigh et al. 2014). However, it is unclear over what timescales such differential dispersal mechanisms can take place; it seems unlikely that the speciation, extinction or range change processes which explain broader patterns of testate amoebae diversity and distribution (Fernández et al. 2016) could have operated over sub-Holocene timescales.

Testate amoebae are predominantly asexual organisms. Alongside *Trinema lineare*, *Corythion orbicularis* and *Corythion delamarei*, *C. dubium* is a rare exception, alternating between asexual binary fission and isogamic copulation of trophozooids during the life cycle (Sukhanova and Yudina 1990; Yudina and Sukhanova 2000). No research into the reproductive processes exhibited in *C. constricta* has been undertaken. It has been suggested, based on other taxa within the genus, that *Corythion* are a genus of obligate sexual organisms (Lahr et al. 2011). This allows, in principle, the biological species concept to be applied to questions of *Corythion* taxonomy; however, this would require molecular and/or behavioural data, which are currently largely unavailable.

Further Work and Palaeoecological Applications

Controlled laboratory culture experiments can allow for unequivocal assessment of the influence of single selected environmental variables on testate populations (e.g. Wanner 1995) as well as provide material for

molecular analysis, but in this instance, no viable testate amoebae could be cultured from, and no intact cells were observed in, these samples or additional samples requested from other research teams. The logistical issues associated with working in Antarctica are numerous and field site access is often restricted such that resampling is not an option. In addition, opportunities for direct measurement of environmental data at the time of biological sample collection are minimal and on-going field measurement is not possible owing to the logistic practicalities. There are thus a number of key limitations on this study. First, considerable size variation, often coupled with other characteristic morphological differences, was observed within *Corythion* spp. on the AP, but these morphologies were not consistent and a continuous and broadly linear size distribution (test length vs. width), suggesting that the taxon should be considered morphologically plastic. There is also no method of partitioning this variation between phenotypic and genotypic. A lack of molecular investigation into this taxon, coupled with these subtle morphological inconsistencies, prevents further formalised varietal subdivision of *C. dubium*. Consistency in the morphological characteristics of cluster 1 allows for confidence in referring it to *C. constricta*. However, it is clear that very little is known about this taxon, including its potential distribution, and analysts should be made more aware of its existence. If it can be confidently separated from *C. dubium* in other regions, then molecular work would be merited to better understand its phylogeny.

Whilst progress made via molecular analysis is important in gaining an improved understanding of testate amoebae phylogeny, morphometric analysis, as carried out in this study, forms the vital basis of phenotypic analysis (Bobrov et al. 2004) and is crucial in the development of morphological taxonomy that can be practically put to use in a range of ecological and palaeoecological contexts. Furthermore, the relative influence of genetic inheritance and phenotypic plasticity in determining test morphology varies by both taxon and trait, and so a morphology-focused taxonomy remains key (Mulot et al. 2017). Testate amoebae are most commonly used to reconstruct past water table dynamics in peatlands (Amesbury et al. 2016; Swindles et al. 2015; Van Bellen et al. 2014), but have also been employed to assess ecosystem health (Fournier et al. 2012) and water quality in lakes (Roe et al. 2010), and to reconstruct sea-level from salt marsh deposits (Barnett et al. 2017). Studies such as these which involve a large amount of routine counting, must by default employ taxonomy based on morphological criteria, as practically identifiable using light microscopy (Charman 1999; Mitchell et al. 2008).

As well as a clear delineation between *C. dubium* and *C. constricta* in surface samples, down-core analysis of sub-fossil testate amoebae from recent sections of two monospecific (*C. aciphyllum*) moss bank sequences from Ardley Island on the AP, where testate amoebae concentration was high, revealed relative fluctuations between the two *Corythion* morphospecies identified, further supporting the taxonomic legitimacy of delineating between these two taxa (Fig. 11). This relative variation implies the influence of environmental driver(s), which this study has been unable to identify owing to the difficulty of obtaining complementary environmental data as a result of the logistical and practical constraints associated with working on the AP. The relative paucity of records of long-term palaeoclimate and biological change on the AP means that every effort should be made to extract all available palaeoecological information from these records. To achieve this sufficiently detailed, accurate and practical taxonomy is vital (Kosakyan et al. 2016a), particularly in areas, such as the AP, where species diversity is low (Royle et al. 2016). There is also a need for the continued study of *C. constricta*, its distribution and ecological/environmental preferences, on the AP but also more broadly in other areas of the world.

The quality and accessibility of high-resolution light and scanning electron microscopic imaging equipment and software, coupled with the advent of online electronic keys and taxonomic websites, should now facilitate the creation, organisation and availability of comprehensive taxonomic keys based on morphology for practical application in ecological and palaeoecological studies (cf. Kosakyan et al. 2016a; Mulot et al. 2017). Particular focus is required for genera, such as *Corythion*, which have been comparatively under examined in the taxonomic literature.

Conclusions

This study examined morphological variation in the testate amoeba genus *Corythion* on the AP, finding the presence of two morphospecies. *Corythion constricta* was recorded on the AP for the first time, suggesting that it may have previously been overlooked and misidentified as the globally ubiquitous *C. dubium*. The absence of *C. constricta* in widely used taxonomic guides may have led to the misidentification of this taxon and analysts globally should be aware of this. Little is known about the ecological preferences of *C. constricta* and more targeted work must be undertaken to rectify this.

A southerly trend of decreasing average test size in *C. dubium* along the AP suggests that the taxon exhibits an adaptive polymorphism, although the precise environmental drivers of this remain unclear. It seems likely that a complex interaction of drivers, including the availability of ambient energy (cf. Fernández et al. 2016), are responsible for this trend. Further work into morphological variation in *C. dubium*, and *Corythion* more broadly, is clearly needed in other areas of the world, alongside molecular analyses to evaluate the potential for cryptic diversity within the genus. However, despite increasing numbers of molecular studies focussing on testate amoebae, it remains unclear which morphological characteristics are important for taxonomic distinction (Oliverio et al. 2014) and so it is likely to remain difficult to reliably subdivide *C. dubium sensu lato* for practical identification by light microscopy.

More broadly, this study demonstrates that a considerable amount of taxonomic uncertainty and potentially cryptic diversity may exist in genera outside those that traditionally attract the most phylogenetic attention (e.g. Hyalospheniidae). Many molecular studies focus on taxa with apparently restricted geographical distributions, and other more ubiquitous taxa and genera, such as *Corythion*, are under-represented in such studies. However, whilst molecular studies of testate amoeba are important to further our understanding of these diverse polyphyletic organisms, and the broader biogeographical themes associated with microbial ecology, the importance of morphological studies for the practical application of taxonomy to ecological and palaeoecological studies should not be underestimated or ignored. We advocate a parsimonious approach that recognises genetic diversity in testate amoebae but also examines and develops accurate morphological divisions and descriptions suitable for application using light microscopy.

Methods

Study sites and sampling: All samples for this study were collected in January 2012 and 2013, apart from those from Signy Island, which were collected in 2009. Sixty one surface samples were taken from moss banks on six islands from across the western AP (Figure

1), spanning a latitudinal gradient of c. 600 km and divided between Signy Island (60.7°S), Elephant Island (61.1°S), Ardley Island (62.2°S), Barrientos Island (62.4°S), Norsel Point (64.7°S) and Green Island (65.3°S). Two short cores were taken from *Chorisodontium aciphyllum*-dominated moss banks on Ardley Island. Preparation of surface and sub-fossil testate amoebae samples followed the same methodology. Testate amoebae were isolated from 2 cm³ organic matter samples using standard techniques (Charman et al. 2000; Booth et al. 2010) and counts were completed on the sieved 300 to 15 µm fraction.

Light microscopy and morphometry: Whilst testate amoebae are morphologically complex, their identification using standard microscopic techniques often relies on examination in broad lateral view and so length and breadth of test are the most useful measurements to examine for practical use. *Corythion* spp. individuals were randomly selected for examination and measurement using light microscopy. Four morphometric features (length of test, breadth of test, width of test aperture, height of test aperture) were measured at 400 x magnification using Zeiss AxioVision microscopy software. Five hundred individuals were measured at random from surface samples taken from Signy, Elephant, Ardley, Barrientos and Green Islands. 250 individuals were measured from Norsel Point, where overall testate concentrations were lower.

Scanning electron microscopy: Samples were diluted with distilled water in petri dishes. A small number of individual *Corythion* spp. tests were picked from the sample using a glass pipette and agglutinated debris was removed as far as possible using gentle agitation. Clean tests were then transferred to coverslips, dried and sputter-coated with platinum and examined using a Zeiss Sigma scanning electron microscope. Photos were then selected based on their quality for illustrative purposes.

Cluster analysis: Cluster analysis serves to classify multivariate datasets of previously unknown structure into meaningful groups (Fraley and Raftery 2002). Clustering methods based on probability models are increasingly preferred over heuristic methods (e.g. k-means and hierarchical clustering algorithms) (Fraley et al. 2007), as they provide an estimation of the number of clusters present and a method of identifying the most appropriate clustering method, systematically and objectively. The mclust package (Fraley et al. 2012) applies a series of Gaussian finite mixture models to the dataset by an Expectation-Maximisation algorithm for maximum likelihood estimation (Banfield and Raftery 1993). The method optimises the number of components (i.e. clusters) across each of the 14 covariance models, which vary in the shape, volume and orientation of their parameters (see Fraley et al. 2007, 2012 for full details of each model). Model performance is assessed using Bayesian Information Criterion (BIC) approximation (Schwarz 1978), a combination of Bayes factor and posterior model probabilities (Kass and Raftery 1995), with higher BIC indicating lower average classification uncertainty and subsequently a better fit to the data. In this study, mclust was performed on the full dataset of four morphometric variables (test length, test breadth, aperture width, and aperture height) in R version 3.3.1 (R Core Development Team 2016). The 4-component VEE model provided the best fit, with a BIC value of -51280.16 (Supplementary Material Fig. A.1).

Principal components analysis: Principal components analysis was performed on the full dataset (2750 individuals) following normalisation, following standard methodologies in PAST v. 3.14 (Hammer et al. 2001).

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Figure legends

Figure 1. Location of moss bank sites used in this study along the western Antarctic Peninsula.

Figure 2. Length vs. breadth measurements for *Corythion dubium* from six sites across the Antarctic Peninsula region, presented in latitudinal order (top-bottom, North-South).

Figure 3. Distribution of morphometric measurements for *Corythion dubium* from six sites across the Antarctic Peninsula region, presented in latitudinal order (left-right, North-South).

Figure 4. Matrix of biplots showing (top right panels) the components (clusters) obtained from the 4-component VEE model (top right) performed on four morphometric variables (test length, test breadth, aperture width, aperture height) and (bottom left panels) the uncertainty in converting conditional probability from Expectation-Maximisation to the classifications obtained from the 4-component VEE model (see Fraley et al. 2007, 2012 for full details of each model). All units represent μm . Green = cluster 1, blue = cluster 2, purple = cluster 3, red = cluster 4. For references to colour, the reader is referred to the online version of this article. For panels G-L, dot size corresponds to the level of uncertainty, with larger dots indicating greater uncertainty in classification.

Figure 5. Principal components analysis performed on four morphometric variables (test length, test breadth, aperture width, aperture height) then coloured by cluster, as identified in mclust, and 95% ellipses added. Green = cluster 1, blue = cluster 2, purple = cluster 3, red = cluster 4. For references to colour, the reader is referred to the online version of this article.

Figure 6. Biplot demonstrating the approximate boundaries between clusters (dashed lines), with the delineation identified as those above and below $45 \mu\text{m}$. Solid line indicates linear regression of clusters 2, 3 and 4.

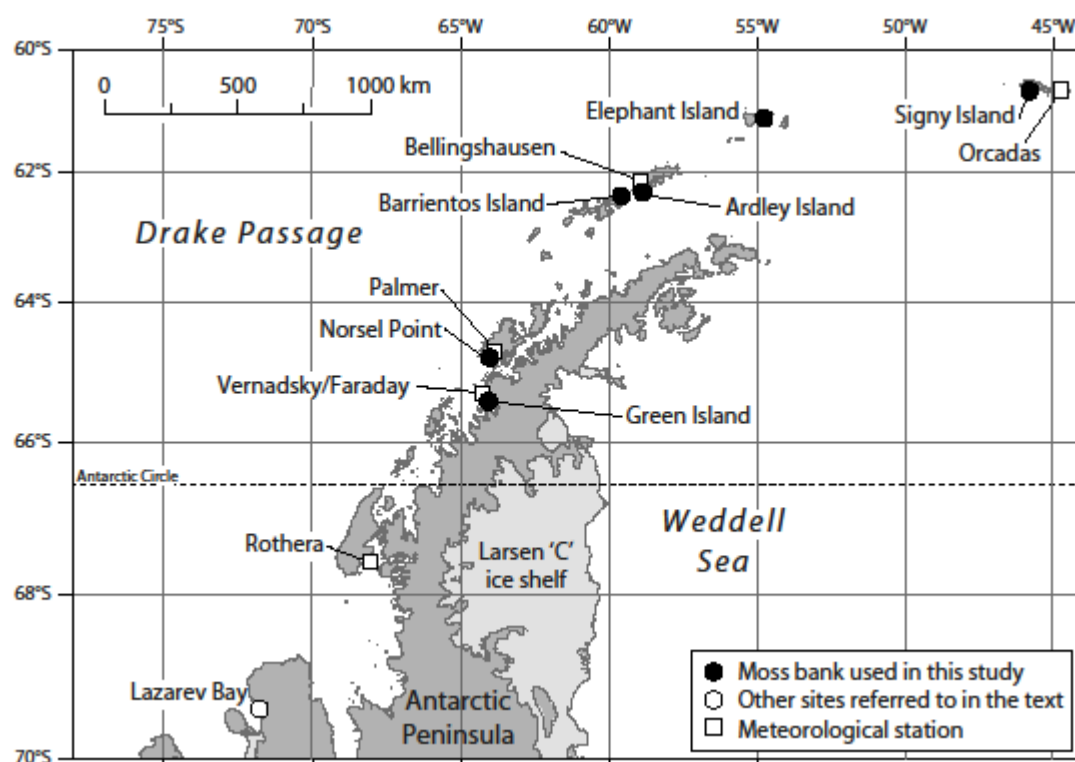
Figure 7. Scanning electron microscope images of *Corythion* tests, **A)** individuals from the two main clusters (cluster 1, $>45 \mu\text{m}$; and clusters 2, 3 and 4, $<45 \mu\text{m}$) from Green Island; examples of morphometric plasticity along the length-breadth gradient within the smaller group (clusters 2, 3 and 4) with individuals from Green Island (**B, D**) and Elephant Island (**C**).

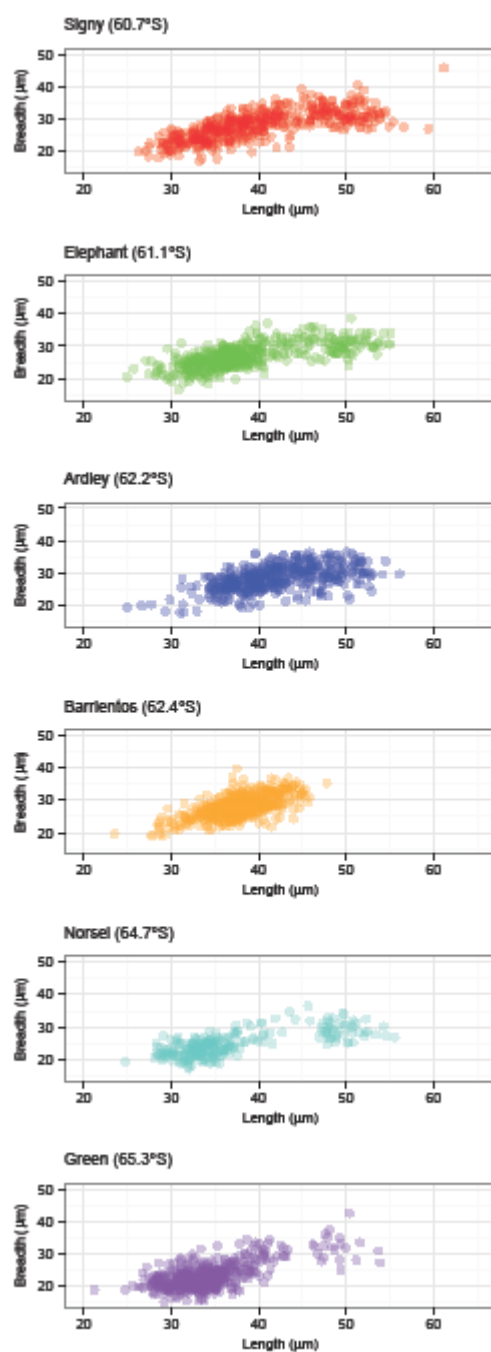
Figure 8. Light microscope image of *Corythion constricta* test from Elephant Island. *Corythion constricta* can be distinguished from *C. dubium* by its larger size ($> 45 \mu\text{m}$) and pronounced pre-apertural rim (indicated by arrow). The margin of this rim is normally 2-3 plates in thickness, compared to the single plate thickness typically characteristic of *C. dubium*.

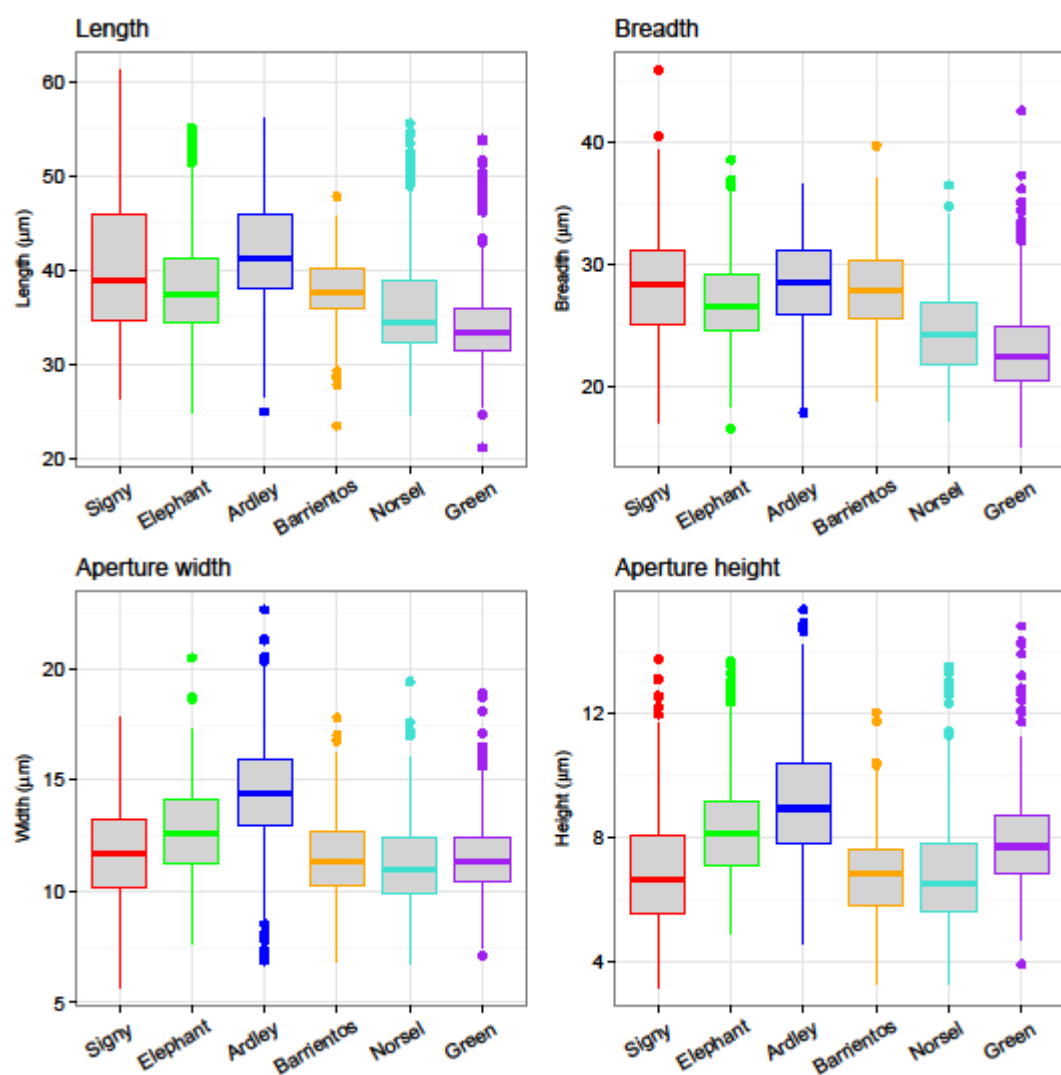
Figure 9. Length vs. breadth measurements for *Corythion dubium* from six sites across the Antarctic Peninsula region as seen in Figure 2 but coloured by cluster and presented in latitudinal order (top-bottom, North-South). Green = cluster 1, blue = cluster 2, purple = cluster 3, red = cluster 4. For references to colour, the reader is referred to the online version of this article.

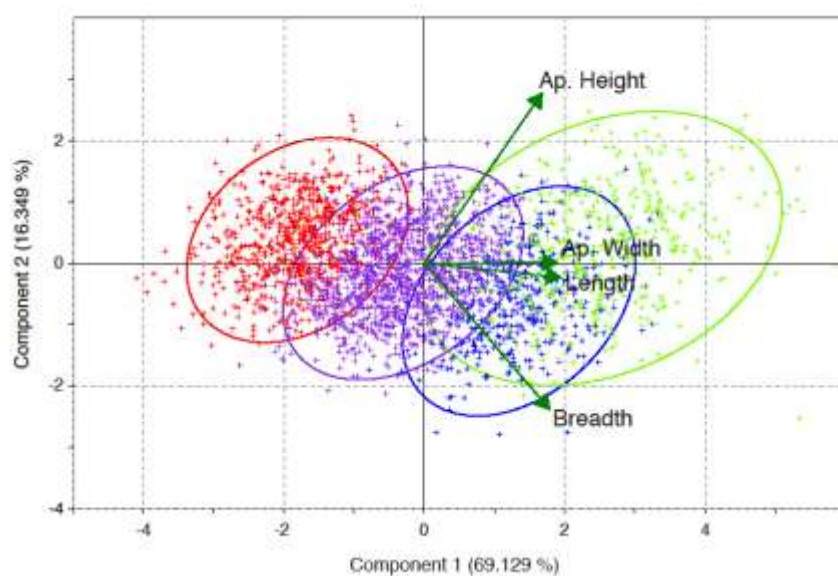
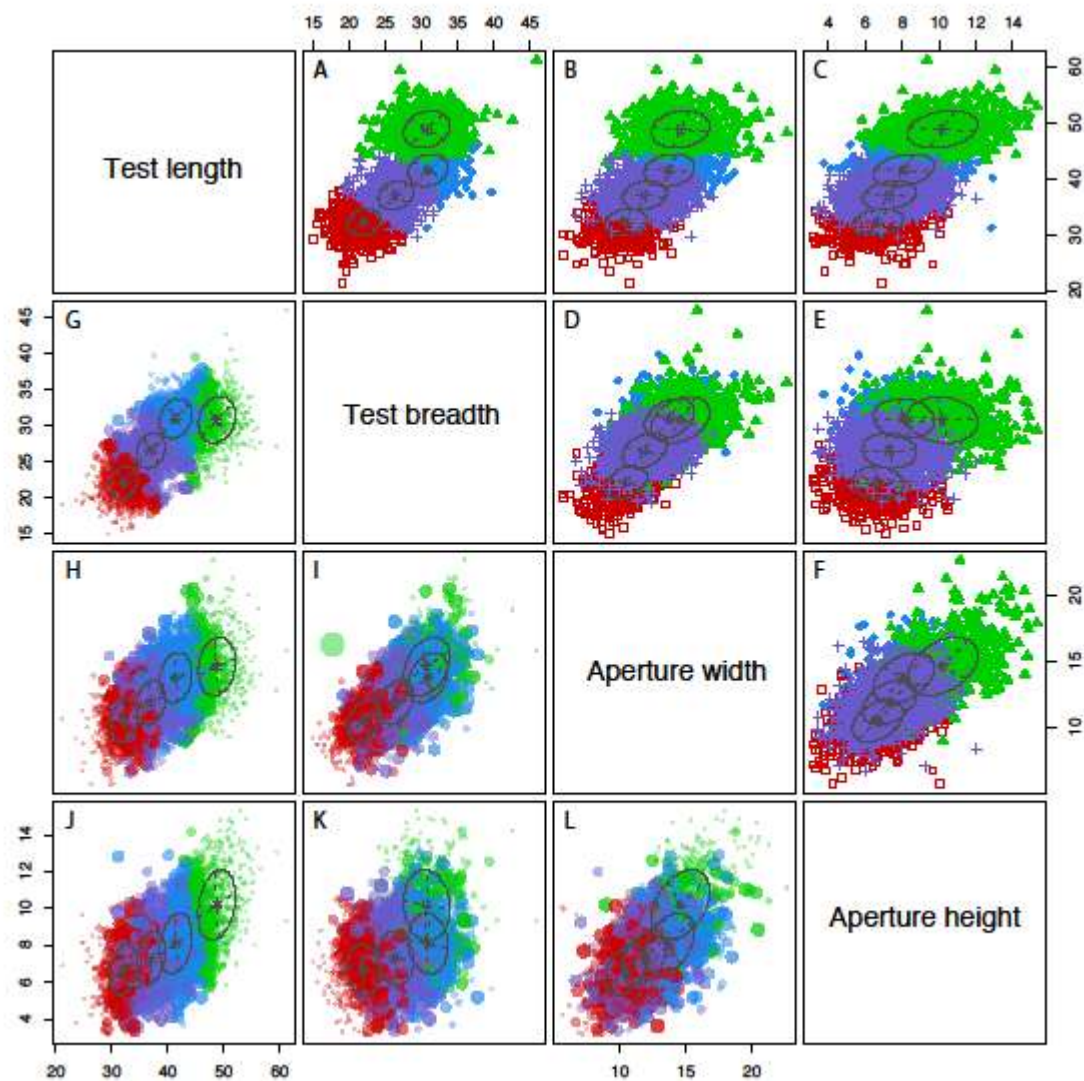
Figure 10. Distribution of morphometric measurements for *Corythion dubium* from samples dominated by different moss types (C = *Chorisodontium aciphyllum*, P = *Polytrichum strictum*, CP = *Chorisodontium/Polytrichum*) from sites across the Antarctic Peninsula region.

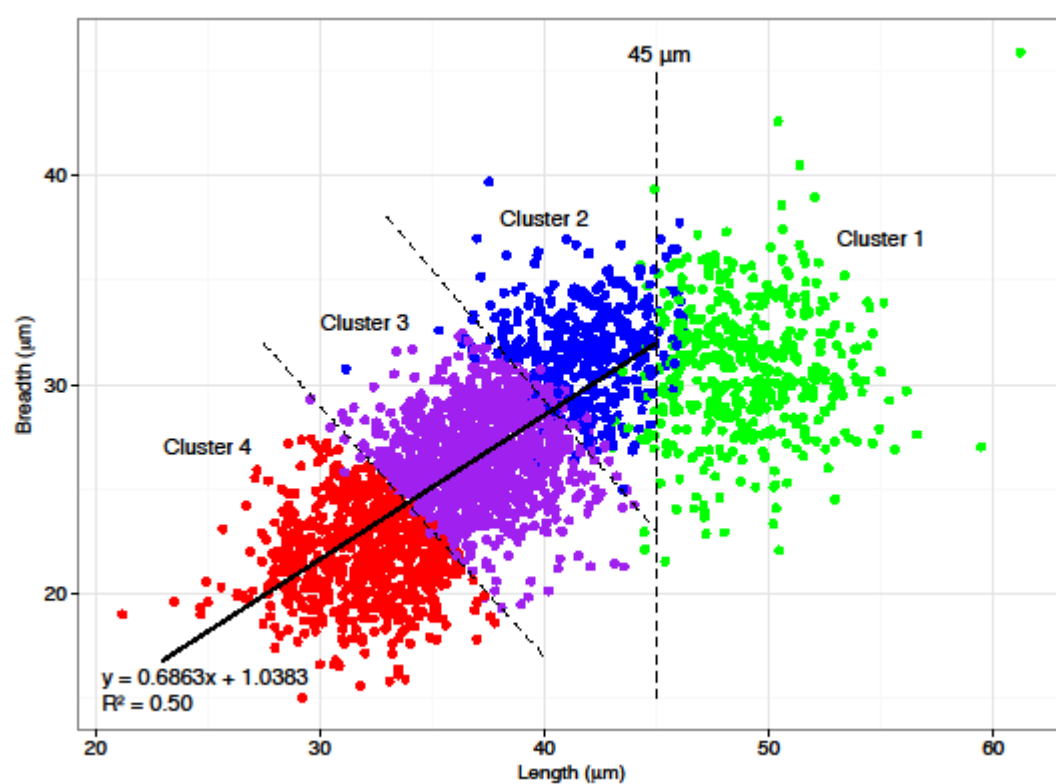
Figure 11. Down-core variation in *C. dubium* and *C. constricta* proportional contribution in two cores taken from Ardley Island as a percentage of the total *Corythion* present.

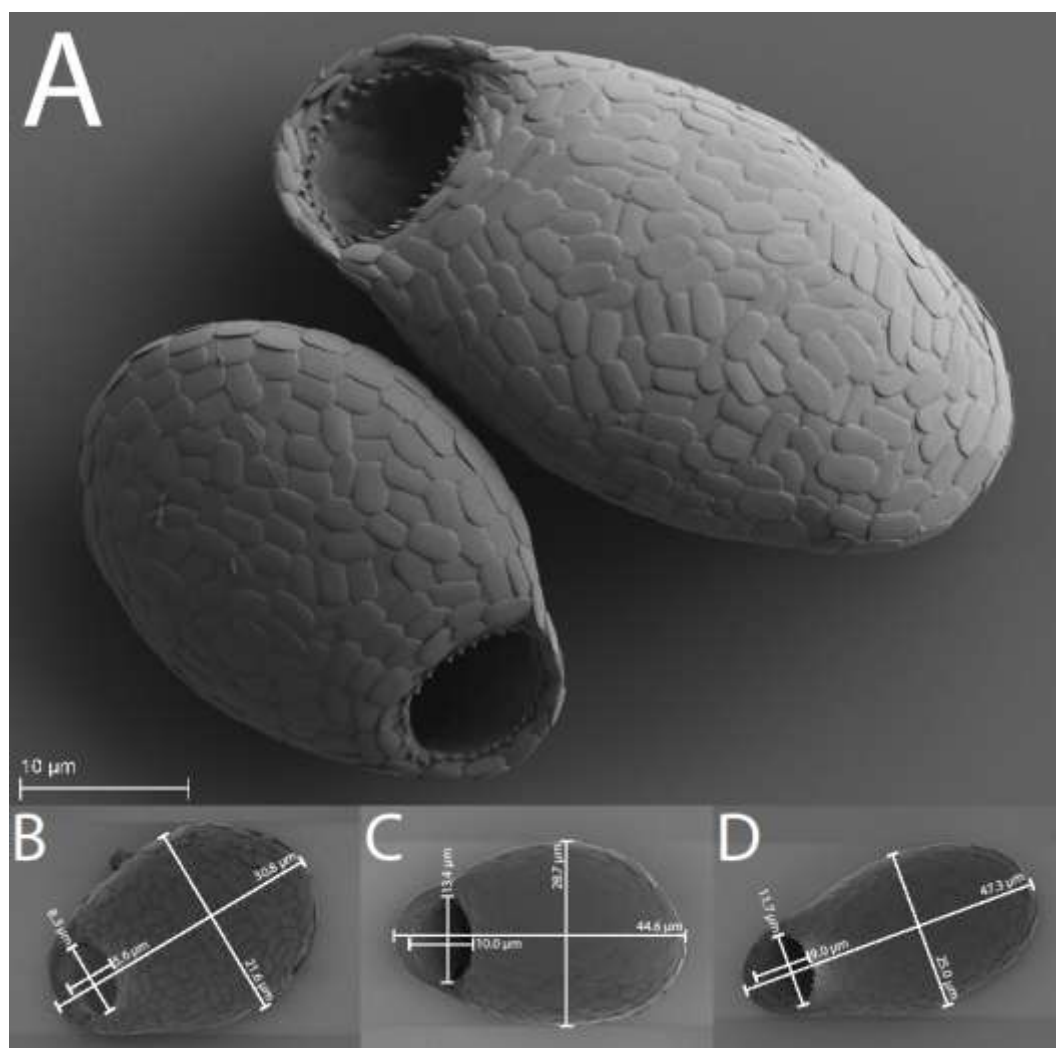


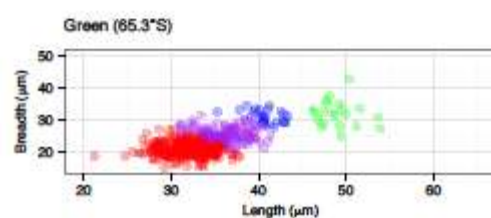
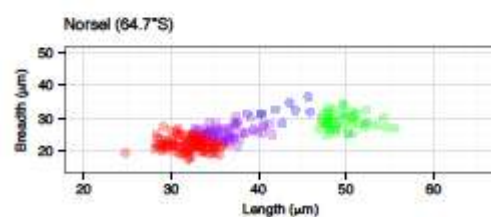
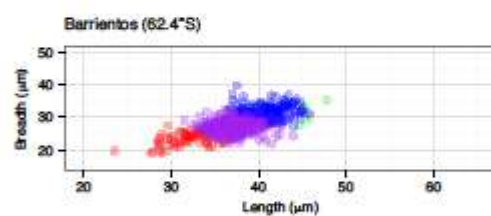
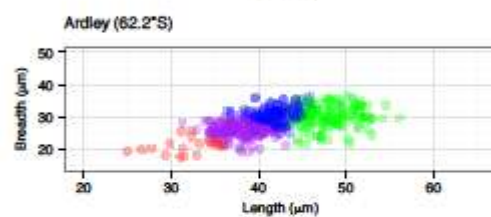
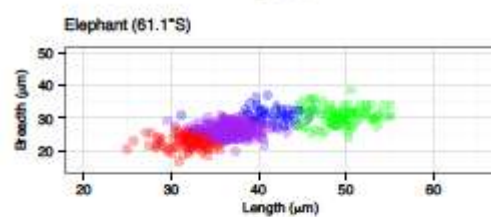
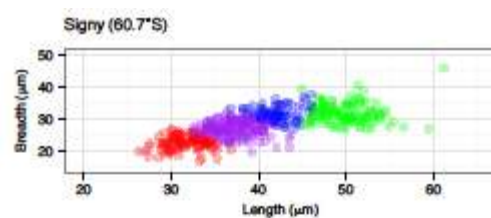


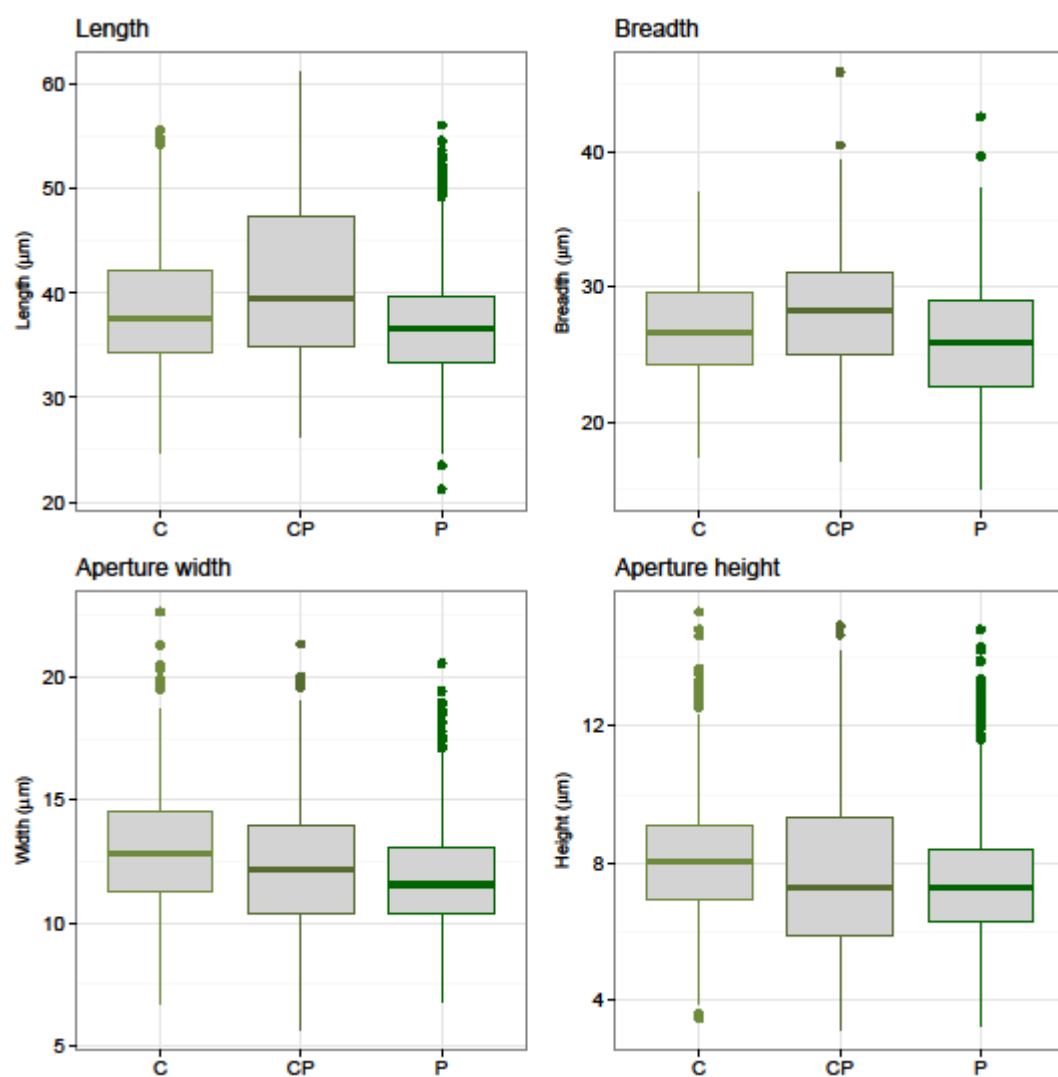












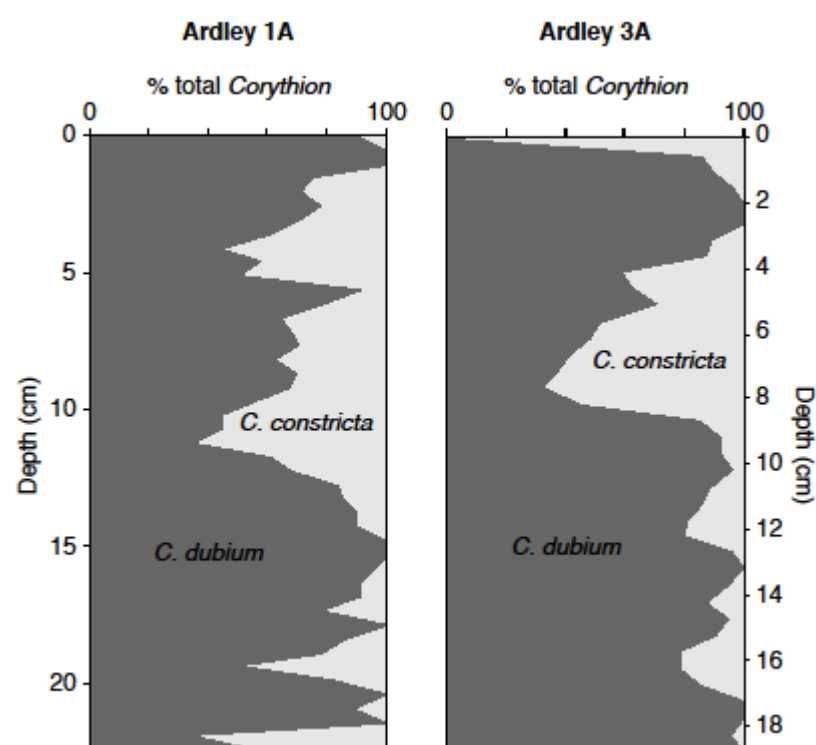


Table 1. Summary of morphometric characteristics of *Corythion* spp. individuals from six locations across the Antarctic Peninsula

Site	<i>n</i>	Test length (µm)				Test breadth (µm)				Aperture width (µm)		
		Min.	Max.	Mean	S.D.	Min.	Max.	Mean	S.D.	Min.	Max.	Mean
Signy	500	26.33	61.19	40.11	7.05	17.11	45.91	28.16	4.31	5.70	17.81	11.73
Elephant	500	24.92	55.11	38.69	6.16	16.35	38.59	26.95	3.57	7.67	20.48	12.71
Ardley	500	24.99	56.12	41.94	5.23	17.82	36.57	28.45	3.67	6.87	22.64	14.22
Barrientos	500	23.50	47.80	37.75	3.56	18.90	39.71	27.96	3.46	6.80	17.79	11.47
Norsel	250	24.67	55.60	36.87	6.90	17.14	36.50	24.68	3.66	6.73	19.42	11.22
Green	500	21.20	53.90	34.18	4.62	15.00	42.60	23.17	3.94	7.10	18.90	11.49
TOTAL	2750	21.20	61.19	38.38	6.14	15.00	45.91	26.73	4.27	5.70	22.64	12.26

Table 2. Summary of morphometric characteristics of clusters identified in the dataset using mclust. Cluster 1 has been identified as *Corythion constricta* and clusters 2, 3 and 4 as *C. dubium*.

Cluster(s)	<i>n</i>	Test length (µm)				Test breadth (µm)				Aperture width (µm)		
		Min.	Max.	Mean	S.D.	Min.	Max.	Mean	S.D.	Min.	Max.	Mean
1	448	43.04	61.19	49.12	2.74	21.53	45.91	30.78	3.22	8.96	22.64	14.65
2, 3, 4	2302	21.20	46.16	36.29	4.10	15.00	39.71	25.95	3.99	5.70	18.62	11.80
(2)	411	31.15	46.16	41.75	2.12	25.00	39.71	31.55	2.27	8.30	18.62	14.01
(3)	1160	29.59	43.98	37.13	2.23	19.33	32.43	26.56	2.17	6.68	17.07	11.90
(4)	731	21.20	37.80	31.89	2.30	15.00	27.40	21.82	2.14	5.70	14.60	10.39