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1 # The genetic diversity, morphology, biogeography, and taxonomic
2 # designations of *Ammonia* (Foraminifera) in the Northeast
3 # Atlantic
4

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25 **Abstract**

26 The genetic diversity, morphology and biogeography of *Ammonia* specimens was investigated
27 across the Northeast (NE) Atlantic margins, to enhance the regional (palaeo)ecological studies
28 based on this genus. Living specimens were collected from 22 sampling locations ranging from
29 Shetland to Portugal to determine the distribution of *Ammonia* genetic types across the NE
30 Atlantic shelf biomes. We successfully imaged (via scanning electron microscopy, SEM) and
31 genotyped 378 *Ammonia* specimens, based on the small subunit (SSU) rRNA gene, linking
32 morphology to genetic type. Phylogenetic analyses enabled identification of seven genetic types
33 and subtypes inhabiting the NE Atlantic margins. Where possible, we linked SSU genetic types
34 to the established large subunit (LSU) T-type nomenclature of Hayward et al. (2004). SSU

genetic types with no matching T-type LSU gene sequences in GenBank were allocated new T-numbers to bring them in line with the widely adopted T-type nomenclature. The genetic types identified in the NE Atlantic margins are T1, T2, T3, T6, and T15, with both T2 and T3 being split further into the subtypes T2A and T2B, and T3S and T3V respectively. The seven genetic types and subtypes exhibit different biogeographical distributions and/or ecological preferences, but co-occurrence of two or more genetic types is common. A shore-line transect at Dartmouth (South England) demonstrates that sampling position on shore (high, middle or low shore) influences the genetic type collected, the numbers of genetic types that co-occur, and the numbers of individuals collected. We performed morphometric analysis on the SEM images of 158 genotyped *Ammonia* specimens. T15 and the subtypes T3S and T3V can be morphologically distinguished. We can unequivocally assign the taxonomic names *A. batava* and *A. falsobeccarii* to T3S and T15, respectively. However, the end members of T1, T2A, T2B and T6 cannot be unambiguously distinguished, and therefore these genetic types are partially cryptic. However, we confirm that T2A can be assigned to *A. aberdoveyensis,* but caution must be taken in warm provinces where the presence of T2B will complicate the morphological identification of T2A. We suggest that T6 should not currently be allocated to the Pliocene species *A. aomoriensis* due to morphological discrepancies with the taxonomic description and to the lack of genetic information. Of significance is that these partially cryptic genetic types frequently co-occur, which has considerable implications for precise species identification and accurate data interpretation.

Keywords: *Ammonia;* genetic types; morphometrics; biogeography; taxonomy

1. Introduction

Ammonia is amongst the most abundant and diverse genera of benthic foraminifera worldwide, with possibly as many as 25-30 biological species (Hayward et al., 2004). They occur in the most marginal marine environments with >80% mud/silt, from salt marsh and estuaries to subtidal habitats. Although members of the group are able to cope with the broad range of salinities, oxygen levels and temperatures associated with these habitats, they appear absent from the colder high latitudes (Murray, 1991, 2006). Coastal margin benthic foraminifera, including *Ammonia*, are used in a variety of (palaeo)environmental studies such as monitoring pollution (e.g., Le Cadre and Debenay, 2006; Frontalini and Coccioni, 2008; 2011; Foster et al., 2012; Jorissen et al., 2018), determining sea level changes over time (e.g., Gehrels et al., 2005; Horton and Edwards 2006) and also as proxies in palaeoclimate reconstructions (e.g., Sejrup et al., 2004; Groeneveld and Filipsson, 2013; Dutton et al., 2015; Groeneveld et al., 2018). In addition, since *Ammonia* is easy to collect and culture, it is routinely used for

70 laboratory experiments (e.g., de Nooijer et al., 2009; Keul et al., 2013; Toyofuku et al., 2017).
71 Such studies require a sound understanding of the species concept, since inaccuracy in
72 determining species would result in invalid ecological and biogeographic data application.
73 Further, modern palaeoproxy calibrations are often species-specific (e.g., Rosenthal et al.,
74 1997; Elderfield et al., 2006; Healey et al., 2008), and it is therefore critical to establish and
75 apply the species-specific calibrations to the correct species.
76
77 In foraminifera, taxonomists primarily utilise the morphological characteristics of the test to
78 classify taxa and describe morphospecies (e.g., Loeblich and Tappan, 1987). Despite rigorous
79 taxonomic descriptions and revisions (Ellis and Messina, 1940 and supplements), the
80 inconsistent application of species names and associated synonyms (Boltovskoy and Wright,
81 1976; Haynes, 1992; Pawlowski and Holzmann, 2008) remains a major problem for studies
82 using benthic foraminifera. This inconsistency is a cause of particular confusion between
83 members of the genus *Ammonia*, as they exhibit high morphological variation. Whether this
84 variation is a result of ecophenotypic traits or species differences has generated much debate
85 (for a comprehensive review see Holzmann, 2000 and references therein) and highlights the
86 problems of relying solely on morphological traits for species designation. However, by
87 utilising a combination of molecular characterisation and morphological traits, it is possible to
88 distinguish some of the morphological boundaries that separate the genetic types of *Ammonia*.
89 Globally to date, genetic characterisation of the large sub-unit (LSU) rRNA gene, herein
90 referred to as the LSU, has revealed 14 genetic types within *Ammonia*, that exhibit varying
91 degrees of morphological distinction (Pawlowski et al., 1995; Holzmann and Pawlowski, 2000;
92 Hayward et al., 2004; Toyofuku et al., 2005; Schweizer et al., 2011b; Saad and Wade, 2016).
93 For practical application, the difficulty arises in linking these characterised genetic types to
94 previously described *Ammonia* morphospecies. For *Ammonia*, Hayward et al. (2004) have
95 undertaken the morphological comparison between genetically characterised specimens using
96 the LSU and type material. These authors identified 13 *Ammonia* genetic types, designated T1-
97 T13, of which eight were considered to have been described already, and the associated
98 taxonomic names were therefore assigned to them.
99
100 The T-type nomenclature for *Ammonia* is now well established in the literature. For example,
101 a number of studies have utilised the type descriptions and genetic T-type nomenclature of
102 Hayward et al. (2004), to give taxonomic names to morphologically described (Dissard et al.,
103 2010; Nehrke et al., 2013) and to genetically characterised (Schweizer et al., 2011b; Lei et al.,
104 2016) *Ammonia* specimens. However, many contemporary studies still rely on using
105 morphological taxonomic assignments without reference to the taxonomic descriptions
106 supported by molecular evidence as proposed by Darling et al. (2016) and Roberts et al. (2016).

107 Morphological traits used for species distinction can prove erroneous following genetic
108 characterisation and true ecophenotypic morphological characters could remain unrecognised.
109 Incorrect taxonomic assignments in the literature leads to the merging of mismatched data and
110 flawed interpretation and conclusions. These issues demand that a more rigorous taxonomy
111 should be used for *Ammonia*, based on molecular characterisation and morphometric analysis
112 together with a morphological description of the SEM images associated with each individual
113 genetic type (Darling et al, 2016). If genetic types are found to possess differentiating
114 morphological characters, this should be followed by the allocation of the most appropriate
115 taxonomic name by comparison with formal type descriptions. Ideally, morphospecies names
116 should not therefore be placed onto molecular phylogenies, unless both the morphology and
117 genetic type have been linked to a formally named holotype (e.g., Darling et al., 2016; Roberts
118 et al., 2016). Once established, the rigorous taxonomic link provides a better understanding of
119 the true biogeography and co-occurrences of genetic types that can be morphologically
120 discriminated from those that remain cryptic, and of the ecological niches that they occupy.
121
122 While the genetic characterisation of *Ammonia* specimens using the LSU enabled the
123 development of the genetic type nomenclature for *Ammonia* (T-type; Hayward et al., 2004), it
124 is not the principle gene used for the study of molecular diversity and genetic characterisation
125 in microorganisms, which includes the foraminifera. Instead, the small sub-unit (SSU) rRNA
126 gene, herein called the SSU, is the most commonly used marker. For example, curated
127 databases developed for linking DNA to morphologically based taxonomies in eukaryotes, such
128 as the SILVA rRNA database (Quast et al., 2013), the Protist Ribosomal Reference Database
129 (PR^2, Guillou et al., 2013) and the PFR^2 database for planktonic foraminifera (Morard et al.,
130 2015), all use the SSU. SSU sequences allow the discrimination of most foraminiferal species
131 (Pawlowski et al., 2012), and make up the majority of foraminiferal sequences deposited in
132 publicly available databases such as GenBank (http://www.ncbi.nlm.nih.gov/genbank/).
133
134 The main reference databases for the benthic foraminifera are the forambarcoding database
135 (http://forambarcoding.unige.ch/; Pawlowski and Holzmann, 2014) and the 37f database
136 (Pawlowski and Lecroq, 2010; Lecroq et al., 2011). The forambarcoding database is for
137 identification at species level and is based on a partial sequence of six foraminifera-specific
138 hypervariable expansion segments from the 3' end of the SSU (1,000 -1,200 nt), which is used
139 as the "barcode" for foraminifera (Pawlowski and Holzmann, 2014). This database includes
140 only specimens for which both molecular and morphological data are available, although high-
141 resolution SEM images are not always shown. The 37f database is based on a very short
142 fragment (~100 bp), covering the 37f variable region of the SSU. This database allows for
143 taxonomic assignment of environmental DNA sequences amplified via next-generation

144 sequencing methods. Both databases are curated and although being added to continuously, are
145 still lacking total assemblage coverage and hence require additional data.
146
147 The aims of this study were first to gain a more comprehensive understanding of the genetic
148 diversity and biogeography of *Ammonia* within the Northeast Atlantic shelf seas. We sampled
149 living *Ammonia* specimens from 22 locations across the NE Atlantic Ocean margins to establish
150 their biogeographic ranges, to determine their propensity to co-occur and to investigate their
151 potential cryptic nature. All individual specimens were SEM imaged and the range of SSU
152 barcodes determined for each genetic type identified. We subsequently linked these SSU
153 genetic types to the T-type nomenclature already established for *Ammonia*, to avoid multiple
154 and confusing genetic nomenclature. Using an integrated approach, we carried out
155 morphometric analysis on individual, genotyped tests to identify any distinguishing
156 morphological criteria. Where discriminant features were recognised, we described genetic type
157 morphotype profiles and linked them to formally named holotypes. Where morphological
158 features were more gradational between genetic types, the creation of genetic type morphotype
159 profiles were not possible, and we discuss the taxonomic and ecological implications of this.

160 **2. Materials and Methods**

161 ***2.1 Sampling***

162 Within this project, our sampling strategy was to include the wide range of shelf provinces and
163 biomes found within the middle to high latitude regions of the NE Atlantic. The biogeographic
164 classification of the shelf and upper continental slope is shown in Fig. 1, which follows the
165 most recent biogeographic classification produced for the Oslo and Paris Conventions
166 (OSPAR) Maritime Area (Dinter, 2001). We collected samples from 33 major sampling
167 locations ranging from Svalbard to Portugal. Twenty-two yielded *Ammonia* specimens (Fig. 1;
168 Table 1). Samples containing *Ammonia* were collected from intertidal and subtidal habitats of
169 south Scandinavia, the British Isles and the Dutch, French and Portuguese margins. Intertidal
170 samples were collected by taking a mud scraping from the surface sediment including the
171 flocculent layer and seaweeds were brushed in seawater to detach the foraminiferal tests. For
172 comparisons of the *Ammonia* genetic types and abundances along a transect, equal volumes (38
173 cm^3) of surface sediment were taken with a cylinder corer, down to 1 cm, at upper-, mid-, and
174 lower-shore sites. Subtidal samples were collected either by SCUBA diving or by deployment
175 of coring devices. All sediments and seaweeds were stored at 4°C prior to processing.

176 ***2.2 Identification of live specimens, SEM imaging, and DNA extraction and amplification***

177 Live *Ammonia* specimens were identified and processed through SEM imaging of both
178 umbilical and spiral views, DNA extraction, PCR amplification and cloning as described by
179 Darling et al. (2016). Cloning was performed to ensure accurate designation of genetic types,
180 as intra-individual variation is common within *Ammonia* (Pawlowski, 2000). Individuals were
181 given a unique identification number, which was used at each progressive stage of the SEM
182 image, DNA extraction, amplification and sequencing process.

183 ***2.3 Genetic characterisation via sequencing and screening of partial SSU sequences***

184 Sequencing was carried out according to Darling et al. (2016) using a BigDye Terminator v3.1
185 cycle sequencing kit (Applied Biosystems) and an ABI 3070 DNA sequencer (Applied
186 Biosystems). Once we confirmed genetic type boundaries by DNA cloning and sequencing, we
187 adopted two further approaches to speed up genetic characterisation. The first was to use a short
188 sequence only (the first ~100 bp) which sits within the foraminiferal variable region 37/f
189 (Pawlowski and Lecroq, 2010) providing that it clearly defined the genetic type. The second
190 approach was to use a screening method by designing SSU genetic type (S-type) specific
191 primers to use in conjunction with s14F1 in the secondary PCR using the same PCR conditions
192 (Darling et al., 2016), to give products of different sizes depending on genetic type. We
193 designed primers for the most common S-types in our dataset as follows. S1: 5'-
194 acgcacgatacgcatacacaa -3' (product ~ 530bp). S2: 5'- gacacacgcctgtcgttaaac -3' (product
195 ~280bp). S5a required a mix of three primers to account for the intra-individual variation, S5a-
196 1: 5'-gcccgaaggtgcaacgy-3', S5a-2: 5'-cgtgctcgagagcaacgy-3' and S5a-3: 5'-
197 acctccgaagagagcaacgt-3' (product ~100 bp). S6: 5'-gcgagtaccgaaatacgccg-3' (product ~390
198 bp). We confirmed that primers were type-specific by performing PCRs with the correct
199 *Ammonia* genetic type, other *Ammonia* genetic types and with other foraminiferal species.

200 ***2.4 Amplification and sequencing of the partial LSU sequences***

201 In order to compare our findings with previous studies based on LSU sequences (Hayward et
202 al. 2004; Pawlowski and Holzmann, 2008; Saad and Wade, 2016) we assigned a T-type to our
203 samples. We achieved this by searching GenBank for individuals sequenced for both LSU and
204 SSU genes (e.g., Holzmann et al., unpublished; Schweizer et al., 2011a, 2011b). In addition,
205 we sequenced both the partial SSU and the 5' end partial LSU genes of selected specimens
206 across the range of genetic types collected during this study. Amplifications of the partial LSU
207 sequences were performed with the same PCR conditions as the partial SSU (Darling et al.,
208 2016) and with primers 2TA and LO (Pawlowski, 2000) for the primary PCR followed by
209 2TAbis (5'-gatacgcgctaaacttaaaca-3') and L10r (5′-aacgatttgcacgtcag-3′) in the secondary
210 PCR. We aligned partial LSU and SSU sequences from the same individuals in two separate

211 alignments as described in Section 2.5 to link the LSU sequences with SSU sequences and
212 determine the T-types.

213 ***2.5 Phylogenetic analyses based on partial SSU gene sequences***

214 The partial SSU sequences from this study were edited in ChromasPro v1.5 (Technelysium Pty
215 Ltd) and aligned manually in BioEdit v7.0.9.0 (Hall, 1999). To obtain a full global picture of
216 *Ammonia* diversity, we also included into the alignment all *Ammonia* partial SSU sequences
217 present in the GenBank database in April 2015 (Supplementary Table S1). From an alignment
218 of 1,143 nt sites, 904 sites could be unambiguously aligned for phylogenetic analysis. Sixteen
219 potential groupings were identified in the alignment and a selection of full-length sequences
220 representative of each group were chosen for analyses (Table 2). No outgroup was used in order
221 to maximize the number of alignable sites available for analyses; phylogenetic trees were
222 therefore unrooted.
223
224 Phylogenetic trees were constructed using three different methods. A Bio Neighbor-Joining
225 (BioNJ) tree (Gascuel, 1997) was built using Seaview 4 (Gouy et al., 2010) with 1,000 bootstrap
226 (BS) replicates. Maximum likelihood (ML) analysis was performed with 1,000 BS replicates
227 (Felsenstein, 1985) using PhyML (Guindon and Gascuel, 2003) implemented in Seaview 4.
228 Finally, Bayesian analysis (BA) was built with MrBayes 3.2 (Ronquist et al., 2012). Two
229 independent analyses were done at the same time with four simultaneous chains (one cold and
230 three heated) run for 10,000,000 generations, and sampled every 1,000 generations with 2,500
231 initial trees discarded as burn-in after convergence was reached. The posterior probabilities
232 (PP), calculated during the BA, estimated the reliability of internal branches. The evolutionary
233 models selected were Kimura 2 parameters or K2P (Kimura, 1980) for BioNJ, Hasegawa,
234 Kishino and Yano or HKY (Hasegawa et al., 1985) and General Time Reversible or GTR
235 (Tavaré, 1986) for ML. A mixed model was used for BA that sampled across the GTR model
236 space (Huelsenbeck et al., 2004). To correct for among-site variations, the alpha parameter of
237 gamma distribution (Γ), with four rate categories, was calculated by Seaview (HKY+Γ,
238 GTR+Γ) and MrBayes.

239 ***2.6 Morphometric analysis***

240 ***2.6.1 Image preparation and measurement of morphological characteristics***

241 To investigate whether the genetic types could be distinguished based upon their morphology
242 alone, a combination of 25 morphological test characteristics were acquired from 316 SEM
243 images of both the umbilical and spiral sides of 158 individual *Ammonia* specimens (Table 3).
244 The morphological characters measured were primarily derived from Hayward et al. (2004)

with some minor modifications and omissions. For example, morphological characteristics such as foraminiferal test area and test roundness measurements were calculated following the methods set out by Roberts et al. (2016). The range of measurements of each morphological test characteristic within each genetic type is documented in Supplementary Table S2. All morphometric measurements for each specimen are available in Supplementary Data 1.

Specimens were excluded if >10% of the test was obscured/damaged or if the specimen had not been imaged from both the umbilical and spiral views. In situations where <10% of the test was obscured/damaged, an infilling procedure was conducted following the methods of Hayward et al. (2004). The morphological data were standardised by ranging the variation between each character from 0 to 1, following the methods of Hayward et al. (2004).

2.6.2 Multivariate data analysis

An unweighted pair-group method using arithmetic averages (UPGMA cluster analysis; dendro UPGMA, Garcia-Vallve et al., 2010) and principal coordinate ordination analysis (PCO; PAST version 2.17, Hammer et al., 2001) were used to assess the utility of the 25 morphological characters in delineating the genetic types within the 158 specimens processed, without *a priori* knowledge of genetic groupings. A discriminant function analysis (DFA) was calculated from the results of the standardised dataset to establish the key diagnostic criteria that can be used to aid classification of specimens into each genetically distinct group. T3V was excluded from the DFA multivariate classification procedure because only two specimens were available for morphological analysis within this genetic subtype. The robustness of the assignment is assessed through a resampling cross-validation procedure in SPSS v.22. The morphological characteristic of presence of dorsal opening, although the main morphological criterion used to distinguish T3 from T15"by eye" (Fig. 2), was excluded from this analysis because it did not exhibit enough variance between the genetic types.

3. Results

3.1 Genetic characterisation based on the SSU and phylogenetic analyses

In total, 378 *Ammonia* individuals were SEM imaged and genetically characterised in this study using the partial SSU, via either cloning and sequencing, or screening methods (Supplementary Table S3). Of these, 233 individuals were sequenced, of which 59 have been cloned (between 2-12 clones each) to determine the number of genetic types and the degree of intra-individual variation. The remaining 145 specimens were fast screened with S-Type-specific primers (Section 2.3). Altogether, 388 new partial SSU sequences were produced and deposited in the GenBank database (accession numbers: MH124763-MH125150), with supplementary

279 information (e.g., SEM images) deposited in the database "foramBARCODING"
280 (http://forambarcoding.unige.ch). SEM images of representative individuals for each genetic
281 type are shown in Fig. 2.
282
283 For phylogenetic reconstruction, the sequences generated in this study were manually aligned
284 together with 87 other *Ammonia* SSU sequences retrieved from GenBank (see methods,
285 Supplementary Table S1). The sequences separate into 16 discrete groups within the alignment,
286 of which six were identified within the NE Atlantic, one of which was further split into two
287 subtypes. The NE Atlantic groups were assigned the S-type identifiers S1, S2, S3, S4, S5a, S5b,
288 and S6. The remaining ten groups occur outside the study area in Japan, Israel, USA, Cuba,
289 Australia, New Zealand and New Caledonia.
290
291 A total of 73 partial SSU sequences were used for phylogenetic analyses (46 from GenBank
292 and 27 from this study). All sequences used for phylogenetic analyses are listed in Table 2. The
293 evolutionary relationships among *Ammonia* were inferred using the BioNJ method with the
294 K2P model (Fig. 3). The general topologies retrieved using BioNJ, ML-HKY+Γ, ML-GTR+Γ
295 and BA were slightly different (see Supplementary Figs. S1-S3, respectively). These
296 discrepancies can be explained by the low phylogenetic signal resulting from the relatively
297 limited number of informative sites in the dataset. The statistical support for the BioNJ, ML-
298 HKY+Γ and BA trees is shown at the nodes of the BioNJ tree (Fig. 3).
299
300 Sixteen genetic types previously identified in the alignment were also retrieved in the
301 phylogenetic analyses (Figs. 3 and S1-S3). The genetic types represented by more than one
302 sequence formed monophyletic clades with high statistical support (> 85% BS or 0.85 PP),
303 except for S2, which exhibited either low support (BioNJ: 66%, ML-HKY+Γ: 44%) or was
304 paraphyletic with S3 (ML-GTR+Γ, BA). Within the alignment, S5 can be divided into the two
305 subtypes, S5a and S5b. They were given subtype ranks since their sequence differences are
306 significant but small. Further sampling would improve their characterisation, but there is
307 currently not enough phylogenetic signal in the 1,143 nt site alignment used in this study to
308 fully separate them into two discrete phylogenetic clades (Figs. 3 and S1-S3).
309
310 The relationships between the genetic types are more difficult to assess, as the deeper nodes
311 have low support and the branching patterns sometimes vary between analyses (Figs. 3 and S1-
312 S3). Nevertheless, some groupings are more stable than others. S5a and S5b are closely related
313 subtypes and group together (98/42/0.87). They also form a highly supported group
314 (99/85/82/0.88) with S6 in all analyses. The genetic types S2 and S3 are also closely related
315 with high statistical support (100/96/98/1.00). The two clades (S5-S6 and S2-S3) fall on a

316 common but unsupported branch, but cluster closer to each other than to either S1 or S4 in the
317 phylogeny. Genetic types S1 (100/100/1.00) and S4 (100/90/0.99) are both well supported
318 clades in the unrooted tree.

319 *3.2 Linking SSU to LSU sequences and the T-type nomenclature used in the genetic* 320 *characterisation of* **Ammonia**

321 Genetic characterisation of *Ammonia* utilising the LSU has yielded 13 genetic types, designated
322 T1-T13 (Hayward et al., 2004). In order to avoid confusion, since the LSU T-type nomenclature
323 is already established, we have linked our S-types S1-S6 directly to the LSU nomenclature. A
324 GenBank search revealed that 37 individual specimens had previously been characterised for
325 both their LSU and SSU genes (Hayward et al., 2004; Schweizer et al., 2011a, 2011b). Among
326 them, 19 represent the S-types S1, S2, S4, S5a and S6 identified in this study. In addition, we
327 sequenced individual specimens of S-types S2, S3, S4, S5a and S5b for both genes, (LSU
328 accession numbers: MH136606-MH136620) to obtain their equivalent T-type and also to
329 supplement the available GenBank data (Supplementary Table S4).
330
331 Separate alignments of all the SSU and LSU sequences from the same individuals revealed that
332 the same six clades of *Ammonia* can be recognized in both genes. Two SSU genetic types and
333 two subtypes from this study can be directly assigned to a previously defined T-type (Hayward
334 et al., 2004). These convert to S1=T6, S4=T1, and the two subtypes S5a=T3S and S5b=T3V
335 (Table 4). Allocation of a T-type to S2 and S3 is more complex because specimens containing
336 S2 and S3 SSU sequences have both been previously allocated T2 (Table S1). However, the S2
337 specimens incorporate the T2 LSU sequences, whilst on close inspection, those of S3 differ
338 (Supplementary Data 2). Despite being very closely related, the variable units in our S2
339 specimens were not found in the S3 specimens and vice versa indicating genetic distinction.
340 However, S2 and S3 cannot always be separated in phylogenetic analysis (Figs. 3 and S1-S3),
341 since we use a conservative alignment which does not include the variable units which
342 characterise them (Section 2.5). A more comprehensive sample survey with extensive cloning
343 is required to fully understand the relationship between S2 and S3. We have therefore assigned
344 them to subtypes T2A (S2) and T2B (S3) until their relationship can be fully resolved. In
345 addition, we have allocated T14 to a previously undesignated Australian genetic type (Fig. 3;
346 Schweizer at al. 2011a), and finally T15 is allocated to S6. The established T-type nomenclature
347 (Table 4) will be used for all further results and discussion.

348 **subtypesubtype***3.3 Morphological analysis of the* **Ammonia** *genetic types*

349 A combination of 25 morphological test characteristics (Table 3) were determined from the
350 SEM images of both the umbilical and spiral sides of 158 individual *Ammonia* specimens for

351 multivariate data analysis. The range of measurements of each morphological test characteristic
352 within each genetic type is documented in Supplementary Table S2. UPGMA cluster analysis
353 and PCO analysis were employed to assess the utility of morphology as a tool for *Ammonia*
354 classification without *a priori* knowledge of genetic groupings. A DFA was then performed on
355 the dataset utilising our knowledge of the genetic types, to assess the effectiveness of
356 morphological traits in predicting genetic type membership and to identify the diagnostic value
357 of the morphological features analysed.

358 ***3.3.1 UPMGA analysis***

359 The UPGMA cluster analysis demonstrates that genetic types T3S, T3V, and T15 are
360 morphologically distinct from genetic types T1, T2A, T2B, and T6, as they form discrete
361 clusters within the morphology dendrogram (Fig. 4). In comparison, no clear clustering patterns
362 were identified between the less ornate genetic types T1, T2A, T2B and T6, as they exhibited
363 extensive morphological overlap between the individual specimens (Fig. 4).

364 ***3.3.2 PCO analysis***

365 The primary PCO analysis demonstrates similar results to the UPGMA cluster analysis. Genetic
366 types T3S and T3V can clearly be distinguished from genetic types T1, T2A, T2B, T6 and T15
367 in the PCO morphospace (Fig. 5). In addition, despite low numbers of T3V specimens, T3S
368 and T3V can also be separated from each other. However, unlike the UPGMA cluster analysis
369 (Fig. 4), T15 is not separated from the less ornate genetic types within the PCO morphospace
370 (Fig.5).

371

372 In order to clarify the validity of the morphological separation of genetic type T15 within the
373 UPGMA analysis (Fig. 4), a refined PCO analysis was performed. This analysis omitted the
374 specimens from genetic types T3S and T3V because they were clearly separated by the primary
375 PCO analysis and the UPGMA dendrogram (Figs. 4 and 5). The refined PCO analysis illustrates
376 that genetic type T15 specimens form a discrete non-overlapping cluster, clearly distinct from
377 the PCO morphospace occupied by genetic types T1, T2A, T2B and T6 (Fig. 6), in agreement
378 with the UPGMA analysis. This extended multivariate morphological analysis also reveals that
379 no other genetic type can be clearly delineated, as substantial morphological overlap is observed
380 between genetic types T1, T2A, T2B and T6 within the PCO morphospace (Fig. 6). Although
381 it should be noted that whilst specimens of genetic types T1 and T2B are completely
382 encompassed within the morphospace of genetic types T2A and T6, they do not exhibit any
383 overlap with each other (Fig. 6).

384 ***3.3.3 Discriminant function analysis (DFA)***

385 Genetic type T3V was excluded from the DFA multivariate classification procedure, again (see
386 2.6.2) because only two specimens were available for morphological analysis within this
387 genetic type. The DFA reveals that in total 98.1% of *Ammonia* specimens were correctly
388 classified into their genetic type, based upon their morphological test characteristics and that
389 90.4% were correctly assigned after the cross-validation procedure (Wilks: -0.001, significance
390 p: <0.001). From a total of 156 specimens, three *Ammonia* specimens were misclassified in the
391 DFA, and 15 specimens were misclassified in the cross-validation analysis (Table 5).
392
393 Genetic type T3S exhibits the highest assignment success based upon morphology, as all
394 specimens were correctly classified in both the DFA and cross validation procedures. In
395 addition, no other genetic types were misclassified into this genetic type (Table 5). Specimens
396 of genetic type T15 also exhibit perfect discrimination in the DFA based upon their test
397 morphology. However, the cross-validation procedure illustrates that four specimens of genetic
398 type T15 were incorrectly classified into other genetic types. This misclassification could be
399 explained by the omission of a key discriminatory variable (presence of secondary dorsal
400 openings) from the DFA, because it did not exhibit variance between the groups. This suggests
401 that even with the exclusion of a key morphological trait, this genetic type can be successfully
402 discriminated from other *Ammonia* genetic types based on its other characteristics of test
403 morphology.
404
405 In contrast, the results of the DFA and cross validation procedure indicate that morphological
406 separation between the less ornate genetic types T1, T2A, T2B and T6 is more challenging.
407 Whilst genetic type T2A exhibited perfect discrimination in the DFA and cross-validation
408 procedure, two specimens of T6 and four specimens of T2B were misclassified into this genetic
409 type. Although the DFA illustrates that 87.5-100% of specimens of genetic types T1, T2B and
410 T6 can be correctly classified, only 25-92% of specimens were classified into their correct
411 groups in the cross-validation procedure. In addition, the misclassification of specimens is
412 evenly distributed between the three genetic types (Table 5). This indicates that the interspecific
413 morphological boundaries determined in this study between genetic types are not discrete and
414 are gradational in nature. However, no morphological overlap was observed between genetic
415 types T1 and T2B, suggesting that it may be possible to separate these genetic types from one
416 another based on morphology. The key diagnostic morphological variables identified by the
417 DFA include a combination of ornamentation and structural features. These are development
418 of thickened calcite on the spiral side (24), development of beads and grooves along the edge
419 of the suture (10, 11), porosity features including pore density and pore diameter (5, 20, 21),
420 degree of thickened calcite on folia (8), the development of radial sutural furrows (23),

421 proloculus diameter (22), and test roundness (17). The morphological traits in brackets
422 correspond to the characters described in Table 3.

423 *3.4 Biogeography, depth and habitat preferences of* **Ammonia** *genetic types in the NE* 424 *Atlantic*

425 The biogeographical distribution of each genetic type identified in this study is described in
426 Table 6 and accompanied by individual distribution maps (Figs. 7 and 8). Depth and habitat
427 preferences for each of the genetic types are described in Tables 1 and 6. At Dartmouth (location
428 18), three different *Ammonia* genetic types (T1, T2A, and T3S) were found in a single sediment
429 sample taken from the lower-shore sampling site (Table 1). To determine whether this was
430 consistent across the whole of the intertidal zone or whether different genetic types dominated
431 different areas of the shore, we collected three sediment samples of equal volume ($38cm^3$) along
432 a transect from the upper-, mid- and lower-shore, to genetically characterize the living
433 *Ammonia* profiles. The results show increasing numbers of individuals and genetic types from
434 the upper- to the lower-shore (Table 1; Fig. 9). On the upper shore, T2A comprised 100% of
435 the *Ammonia* assemblage, but only six *Ammonia* specimens were found in total. On the mid-
436 shore, of 15 specimens, T2A made up 80%, whilst T3S contributed 7% of the assemblage and
437 T1 accounted for 13%. On the lower shore, T2A again dominated the assemblage comprising
438 75% of the 65 *Ammonia* specimens, whilst T3S and T1 made up 22% and 3%, respectively
439 (Fig. 9).

440 4. Discussion

441 The taxonomy of *Ammonia* is still in confusion, although the seminal study by Hayward et al.
442 (2004) has brought some taxonomic order to this globally distributed genus. Nevertheless, the
443 identification of *Ammonia* specimens remains hugely challenging, due to the cryptic or pseudo-
444 cryptic nature of some genetic types and the perceived wide morphological variation in others.
445 We now present a clear overview of the seven genetic types and subtypes of *Ammonia* identified
446 along the NE Atlantic Ocean margins. For each genetic type and subtype we have provided
447 SSU barcodes (Genbank) linked to SEM images (forambarcoding database) enabling us to
448 deliver the first morphometric analysis on a dataset of fully barcoded specimens.
449

450 In agreement with Hayward et al. (2004) and Schweizer et al. (2011a), we demonstrate that
451 genetic subtype T3S and genetic type T15 can be morphologically distinguished. In addition,
452 genetic subtype T3V can also be distinguished by morphometric analysis. However, a larger
453 sample set and further genetic profiling is required to establish this subtype as distinct from
454 T3S. We also provide evidence that the remaining four genetic types/subtypes cannot be

455 robustly delineated 100% of the time with the morphometric analyses performed in this study
456 and, at present, should be considered as cryptic species. However, a semi-automated method to
457 measure the porosity of *Ammonia* tests presented in other studies (Petersen et al., 2016; Richirt
458 et al. in press) may prove an additional and useful tool for their discrimination.
459
460 Following the strict integrated approach proposed by Roberts et al. (2016), it is not possible to
461 assign taxonomic names without adding further potential confusion to the literature. However,
462 the taxonomic allocations made by Hayward et al. (2004) for these genetic types are discussed
463 below. We provide biogeographical distributions of each genetic type within the NE Atlantic
464 margins and ecological information including co-occurrence profiles, which, combined with
465 morphological information, will be helpful in identifying genetic types in the field.
466
467 Whilst the sampling regime employed in this study provides a broad overview of the regional
468 distributional patterns of *Ammonia* in the NE Atlantic, it is important to recognise that it is not
469 exhaustive. For example, some of the biogeographic provinces identified in Dinter (2001) have
470 not been sampled, such as the Warm Lusitanean subprovince and the White Sea. Additionally,
471 the Cool Lusitanean and West Norwegian subprovinces have only been marginally sampled
472 (Fig. 1). Nevertheless, the northern limit of *Ammonia* is known (around 60°N), and the bias
473 concerns mainly the southern region. There is also a sampling bias towards intertidal areas.
474 Consequently, the complete genetic and morphological diversity of *Ammonia* species may not
475 have been fully captured in the subtidal areas of focus in this study. In addition to data from the
476 NE Atlantic margins, we report a small dataset from subtidal sampling undertaken in the
477 western Mediterranean Sea, to supplement a large body of intertidal sampling that has been
478 documented in the region (e.g. references in Supplementary Tables S1 and S5). Despite these
479 limitations, the sampling employed in this study presents the most extensive genetic and
480 taxonomic evaluation of *Ammonia* diversity conducted to date within this region.

481 ***4.1 Genetic characterisation and molecular phylogeny***

482 As discussed, the T-type nomenclature for the genetic types of *Ammonia* is now well
483 established, despite being based on genetic differences in the LSU rather than the SSU, which
484 is the common practise for the other foraminiferal groups (Pawlowski and Holzmann, 2014).
485 Therefore, to avoid multiple and confusing nomenclature, a primary aim of this study was to
486 bring the T-type nomenclature in line with the molecular characterisation of other foraminiferal
487 groups and to generate SSU barcodes for database submission.

488 ***4.1.1* Ammonia *rRNA gene arrays***

489 *Ammonia* genetic types were initially characterised by direct comparison of SSU sequences
490 within a 1143 nucleotide site alignment, of which only 904 bp could be unambiguously aligned
491 for use in phylogenetic analysis. Examination of the unalignable variable regions of the SSU 3'
492 fragments (e.g., Schweizer et al., 2008; Pawlowski and Lecroq, 2010; Weber and Pawlowski
493 2014), showed that five out of the six SSU genetic types identified in the study area contained
494 several different gene copies. However, the variable units observed within and between
495 individuals were unique to each genetic type, including between the closely related subtypes
496 T2A and T2B. In contrast, T3S and T3V have one shared variable unit. The presence of multiple
497 gene copies within the SSU gene variable regions in *Ammonia* is consistent with our data on
498 elphidiid genetic types (Darling et al., 2016), and with work done specifically on a Patagonian
499 *Elphidium* species (Pillet et al., 2012), together with other foraminiferal species belonging to
500 the rotaliids, textulariids and allogromiids (Weber and Pawlowski, 2014). Multiple gene copies
501 were also observed previously in the LSU of *Ammonia* (Holzmann et al., 1996), confirming
502 that this is a common phenomenon in the rRNA gene arrays of the benthic foraminifera. We
503 used a representative set of SSU gene copies to define each genetic type, with the exception of
504 genetic type T1, for which only one gene copy was found within the eight specimens collected
505 in our study area. However, T1 has a cosmopolitan distribution (Hayward et al., 2004) with a
506 wide range of variable units within its SSU sequences (Fig.3; Table S1), and the New Zealand
507 T1 sequence (HE598562) has identical units to those of our T1 sequences, confirming its
508 identity.

509 ***4.1.2 Phylogenetic analyses based on SSU sequences***

510 The *Ammonia* genetic types T1, T2, T3S, T3V, and T6 identified within our study area, were
511 first recognised by Holzmann and Pawlowski (2000). However, we further divided T2 into two
512 subtypes in this study (T2A and T2B), as there was some degree of support for their separation
513 in the SSU NJ phylogeny. The clade T2B is always well supported (BioNJ: 99%, ML-HK:
514 94%, ML-GTR: 96%, BA: 1.00), whereas the clade T2A has either a low support (BioNJ: 66%,
515 ML-HKY: 44%; Fig. 3; Fig. S1), or does not exist (ML-GTR, BA; Fig. S2-S3). The possibility
516 of two potential subtypes of *Ammonia* T2 was mentioned in Holzmann and Pawlowski (2000)
517 and later Weber and Pawlowski (2014) also suspected the presence of an additional genetic
518 type within the T2 clade. The designation T15 was also allocated to the SSU genetic type S6,
519 which was identified within our study area. This genetic type had previously been
520 morphologically identified as the species *A. falsobeccarii* (Rouvillois, 1974; Schweizer et al.,
521 2011a). Six of the *Ammonia* SSU genetic types (T1, T2A, T2B, T3, T6, and T15) were well
522 supported in the NJ phylogeny (Fig. 3). However, the very closely related genetic subtypes T3S
523 and T3V were not well supported, as the SSU phylogenies produced from our conservative
524 alignment do not fully resolve them (Supplementary Data 3). Nevertheless, they show sufficient

525 difference in their variable regions to be considered subtypes, and these sister genetic types
526 were already split and allocated to T3S and T3V by Hayward et al. (2004) based on their LSU
527 sequences (See Supplementary Data 2 for LSU alignment of T3S and T3V). Investigation of
528 the hypervariate rRNA internal transcribed spacer (ITS) region of these two genetic types, in
529 combination with additional T3V specimens sampled and cloned from other locations, may
530 shed more light on their phylogenetic relatedness.
531
532 One divergent clade within the phylogenetic tree (Fig. 3) includes two sequences from Lizard
533 Island on the East coast of Australia (Schweizer et al., 2011b). We have designated this genetic
534 type T14, since it has not previously been assigned. Interestingly, it has not yet been identified
535 across the Coral Sea in New Caledonia where T1, T12 and T13 were all found. However,
536 Hayward et al. (2004) reported six distinctive *Ammonia* morphotypes within the sediments
537 there, suggesting that there are three types still to be sequenced. Whilst it is important to note
538 that some of these morphotypes might be present as a result of post-mortem transport, it is also
539 plausible that T14 is one of these New Caledonia morphotypes that are yet to be sequenced.

540 ***4.2 Morphological discrimination of* Ammonia *genetic types and cryptic diversity***

541 This study is the first morphometric analysis performed on a dataset of fully genotyped
542 specimens including the complete range of *Ammonia* genetic types identified along the NE
543 Atlantic margins. These are T1, T2A, T3S, T3V and T6, which were also analysed by Hayward
544 et al. (2004), plus T2B and T15, which were not analysed in the 2004 study. In total, 316 SEM
545 images from 158 specimens were used in morphometric analyses. Using a range of statistical
546 analyses, we have directly compared the interspecific taxonomic boundaries identified by
547 quantitative morphological analysis, against the seven distinct genetic types and subtypes from
548 the NE Atlantic.

549 ***4.2.1 Morphologically resolved genetic types***
550 Genetic subtypes T3S and T3V can be robustly distinguished from genetic types T1, T2A, T2B
551 and T6, using a combination of structural and ornamental test characteristics (Table 3;
552 Supplementary Table S2). Interestingly, although only limited genetic divergence has been
553 identified between T3S and T3V (Fig. 3; Supplementary Data 2 and 3), they exhibit clearly
554 distinctive morphologies (Figs. 2, 4 and 5), providing substantial support for their potential
555 genetic distinction. T3S can be distinguished based on a combination of morphological
556 characters, including the development of thickened calcite over the spiral central area (Fig. 2).
557 This species also typically exhibits a more pronounced development of the radial sutural
558 furrows than specimens from genetic subtype T3V (Supplementary Table S2). In addition, T3S
559 commonly possesses a number of umbilical bosses (0-3). In contrast, T3V lacks a distinctive

560 umbilical boss. Instead, T3V seems to be distinguishable from T3S by its stronger development
561 of beads and grooved notches on the umbilical side, which sometimes extend all the way to the
562 periphery of the test (as depicted in Fig. 2; Supplementary Table S2). T15 can be discriminated
563 by a single discrete morphological test trait that is the presence of secondary dorsal openings
564 (Fig. 2; Schweizer et al., 2011a), illustrating the effectiveness of morphology as a tool for
565 species delineation in these genetic types.

566 ***4.2.2 Morphologically cryptic genetic types***

567 The remaining four *Ammonia* genetic types (T1, T2A, T2B and T6) have significantly fewer
568 discriminating characteristics. They overlap in the PCO morphospace, in the UPGMA cluster
569 analysis tree, and even though T2A was correctly assigned to its genetic type 100% of the time
570 in the DFA cross validation procedure (Figs. 4, 5 and 6; Table 5), other genetic types (T6 and
571 T2B) were misclassified as T2A. These genetic types exhibit gradational test characteristics,
572 i.e., the morphological boundaries between them are not discrete (Supplementary Table S2).
573 They exhibit the least test ornamentation, possess a broadly rounded periphery and have a
574 similar number of visible test chambers per whorl (Supplementary Table S2).
575

576 Genetic type T6 has the largest average pore diameter (mean diameter 1.0-4.23 μm on the spiral
577 side and 1.39-8.64 μm on the umbilical side, (Supplementary Table S2). However, the average
578 pore sizes of end members of T1, T2A and T2B all overlap with T6, with the exception of T2B
579 on the umbilical side, where average pore size is smaller (there is overlap on the spiral side). In
580 contrast to T6, genetic type T2A commonly has smaller pores (mean diameter 0.51-1.26 μm on
581 the spiral side and 0.33-2.02 μm on the umbilical side), but higher pore density (4-28 pores per
582 100 sq. μm). Hayward et al. (2004) suggested that T2 can be distinguished from T1 and T6 by
583 its small pores. However, our analysis shows significant overlap with other genetic types in the
584 PCO morphospace (Fig. 6), and misclassifies T6 as T2A in the cross-validation procedure
585 (Table 5). In addition, we have split T2 into T2A and T2B in this study, and T2B commonly
586 has the smallest pore diameters (e.g., mean pore diameter 0.39-0.87 μm on the umbilical side).
587 Both genetic types T6 and T2A also sometimes display the development of small pustules along
588 the edges of umbilical sutures (often extending to the periphery) and ornamentation on the folia,
589 which can help to distinguish them from genetic types T1 and T2B, which rarely exhibit these
590 characters. Genetic type T1 typically exhibits slightly lower pore density in contrast to the other
591 three *Ammonia* genetic types (T1: 6-8 pores per 100 sq. μm; T2A: 4.76-29.40 pores per 100 sq.
592 μm; T2B: 6.06-17.35 pores per 100 sq μm; T6: 0.56-11.35 pores per 100 sq. μm; Supplementary
593 Table S2), together with a fissure on the spiral side (Fig. 2), although this feature is not always
594 strongly developed. T1 also rarely possesses a small umbilical boss, being often depressed in
595 the umbilical region and exhibiting very weak to weak secondary calcite on the spiral area

596 (Supplementary Table S2). Interestingly, in the Lagoon of Venice, where both T1 and T2B
597 have been observed, Holzmann and Pawlowski (1997) reported being able to distinguish "with
598 difficulty" the two distinct genetic types, based on pore parameters and test size. Our
599 morphometric analysis supports this, as T1 and T2B do not overlap in the PCO morphospace
600 (Fig. 6). Pore parameters are one of the key diagnostics in morphometric analyses of the less
601 ornate *Ammonia* genetic types (Hayward et al., 2004; this study). Studies using the semi-
602 automated method to measure the porosity (percentage of surface in the measurement frame
603 covered by pores) of *Ammonia* tests (Petersen et al., 2016; Richirt et al., in press) have
604 discriminated T1 and T6 from T2A/B but T2A and T2B remain morphologically
605 indistinguishable. In the literature, porosity is currently explained either by genetic differences
606 (e.g., Morard et al., 2009; Schweizer et al., 2009; Petersen et al., 2016) or ecophenotypic
607 variations (e.g., Glock et al., 2011; Kuhnt et al., 2014; Petersen et al., 2016; Roberts, 2016).
608 However, with the exception of Roberts (2016), those studies promoting ecophenotypic
609 variations to explain porosity differences, used non-genotyped individuals. Hence, in these
610 studies a genetic basis for changes in porosity cannot be ruled out. The work by Roberts (2016)
611 however, does provide some evidence for ecophenotypic variation of pore size. *Ammonia* T6
612 specimens from Hanö Bay (location 7) had significantly larger pore size than T6 specimens
613 from Norfolk (location 13), Laugharne Castle (location 14) and Cardiff (location 17). The major
614 difference between these four sites is that the *Ammonia* T6 specimens were sampled from low
615 salinity (7-13) subtidal waters at Hanö Bay as opposed to intertidal mudflats at all other
616 locations. In addition, the Hanö Bay specimens demonstrated significant signs of etching,
617 which may have contributed to the larger pore sizes, and more studies are required therefore,
618 to determine habitat influence on pore size.
619
620 This study shows that genetic types T1, T2A, T2B and T6 are partially cryptic due to overlap
621 of endmembers. Whilst some key diagnostic morphological variables were identified (Section
622 3.3.3), no diagnostic features were found in this study to consistently delineate between these
623 genetic types. This indicates that the less ornate genetic types are practically cryptic in an
624 applied taxonomic situation. These results underscore the necessity for employing multiple
625 lines of evidence (such as DNA, ecology, morphology, and biogeography) for re-evaluating
626 taxonomic boundaries within this genus, because at present, morphology alone is insufficient
627 for elucidating diversity. This is illustrated by a recent morphometric study focusing on the
628 morphology of sequenced genetic types T1, T2A/T2B together and T6, which has successfully
629 discriminated these three groups on the basis of morphological criteria observable with a
630 stereomicroscope (Richirt et al., in press).

631 ***4.2.3 Comparative morphometric studies in* Ammonia**

632 Hayward et al. (2004) found that all molecular types could be discriminated based on their
633 morphology, although end members were hard to distinguish from each other. Superficially,
634 this appears counter to our findings, as our study suggests that there is morphological overlap
635 between end members that make some genetic types partially cryptic. However, previously
636 unrecognised genetic diversity could account for some of the differences in the morphological
637 boundaries observed between our study and those of Hayward et al. (2004). In particular, the
638 splitting of T2 into T2A and T2B, has increased the difficulty in delineating these small less
639 ornate genetic types. T2B is entirely enclosed within the morphospace of T2A and overlaps
640 with T6 (Fig. 5), and 25% of T2B specimens are misclassified as T2A in the DFA cross
641 validation procedure (Table 5). The interspecific morphological boundaries identified for T2
642 by Hayward et al. (2004) will therefore encompass the morphological characters of T2B.The
643 differences in the morphological boundaries identified between this study and those of Hayward
644 et al. (2004) may also be the product of the different morphological characteristics analysed.
645 For example, this study measured 23 out of the 37 morphological characters originally assessed
646 by Hayward et al. (2004), and a number of these variables were slightly modified. We also
647 utilised computer-aided techniques to standardise the measurements of several morphological
648 characteristics, thereby reducing human subjectivity. One of the morphological characteristics
649 not measured in this study, but measured by Hayward et al. (2004) is the development of the
650 protoforamen. Hayward et al (2004) determined that T1 always possesses a protoforamen that
651 is often strongly developed. Measuring this characteristic might help in discrimination of T1.
652 Nine of the morphological features used by Hayward et al. (2004) that were omitted in this
653 study were due to the unavailability of SEM images taken from the profile aspect of the
654 foraminifera. Therefore, the taxonomic re-evaluation of the morphological boundaries of
655 *Ammonia* presented in our study might not have captured all the key diagnostic traits. For
656 example, Hayward et al. (2004) identified that the profile diameter is a strong diagnostic
657 character, thus the inclusion of this feature in future investigations may help to discriminate
658 between cryptic specimens.

659 ***4.3 Nomenclature and taxonomy***

660 The genetic types defined for *Ammonia* (T1-T15) are thought different enough to be considered
661 as separate species (Holzmann, 2000; Hayward et al., 2004; this study), yet distinct genetic
662 types are not always morphologically discrete (Pawlowski et al., 1995; Holzmann and
663 Pawlowski, 1997; Holzmann, 2000; Hayward et al., 2004; this study). Where morphological
664 variation is observed, the traditional view would have been that they represent ecophenotypic
665 variants of *Ammonia* (e.g., Schnitker, 1974 Jorissen, 1988; Holzmann, 2000 and references
666 therein). However, in agreement with Hayward et al. (2004), the high number of individual

667 specimens genotyped in this study confirms that the morphological differences observed in
668 morphometric analyses are due to genetic distinction and are not a result of environmentally
669 controlled morphological variations. Species names can therefore be confidently allocated to
670 those genetic types that can be morphologically discriminated and match a strict type
671 description. However, several of the genetic types are partially cryptic or pseudo-cryptic (T1,
672 T2A, T2B and T6) and only genetic types T3S, T3V and T15 can be robustly distinguished.

673 ***4.3.1 Allocation of species names***

674 Morphospecies names cannot be confidently allocated to genetic types unless both the
675 morphology and genetic type have been linked to a formally named holotype (Roberts et al.,
676 2016). Ideally live topotypes should also be sampled to complete the picture, but this is not
677 always possible. To overcome this issue, a three-stage approach has been proposed to make the
678 genetic/taxonomic link (Darling et al., 2016; Roberts et al., 2016), which incorporates the
679 following steps. (i) Genetic characterisation with high-resolution imaging of the test, (ii) genetic
680 type delineation by generating a morphotype description produced only from the range of test
681 morphologies associated with the genetic type and (iii) allocation of the most appropriate
682 taxonomic name by linking the genetic type morphotype description to a taxonomic
683 morphospecies description, using only strict morphological criteria. Of those species that can
684 be robustly delineated via morphometric analysis, T3S and T15 can be confidently allocated
685 morphospecies taxonomic names using this three-step method. The allocation of T3V as a
686 distinct subtype or species, and hence the allocation of a species name, requires further analysis
687 of additional specimens to confirm the morphological delineation observed here and to
688 determine the uniqueness of the units of intra-individual variation in the rRNA gene arrays.

689

690 *Genetic subtype T3S description.* – Test relatively large, trochospiral, inflated and usually with
691 lobulate periphery, at least in the last part of the final whorl. Between 8 and 12 chambers in the
692 final whorl. On the spiral side, it typically has pronounced development of sutural furrows along
693 both the radial chamber sutures and the spiral suture. These are usually restricted to the later
694 part of the last whorl, but they are sometimes found almost throughout the last whorl. It has
695 often developed thickened calcite over the spiral central area. Relatively strong development of
696 beads and grooved notches are seen on the umbilical side, sometimes extending to the
697 periphery. Usually it has one large umbilical boss, sometimes up to three, but sometimes
698 lacking.

699

700 In agreement with Hayward et al. (2004) we link genetic type T3S to the morphospecies
701 *Ammonia batava* (Hofker, 1951). Hofker (1951) described this new species (as *Streblus*
702 *batavus*) with the North Sea as type locality (Voorne Island, The Netherlands). Hofker (1951)

703 separated *Streblus batavus* from *Ammonia beccarii* (Linné, 1758), i.e., as a smaller and less
704 depressed form, and he discussed in detail the differences between *Streblus batavus* and the
705 type material of *Ammonia beccarii* (Linné, 1758) from Rimini in the Adriatic, including
706 differences in apertural and internal structures.
707
708 *Genetic type T15 description.* – Test relatively large, trochospiral, inflated and typically with
709 lobulate periphery, at least in the last part of the final whorl. Between 7 and 9 chambers in the
710 final whorl. A typical morphological test trait for this genetic type is the development of
711 secondary dorsal openings where the spiral suture meets the radial chamber sutures. In most
712 specimens these openings are only developed along part of the last whorl, but they are often
713 seen throughout the last whorl and sometimes even along part of the second-last whorl.
714 Relatively strong development of beads and grooved notches are seen on the umbilical side,
715 but these are usually restricted to the central area and not extending to the periphery. There is
716 no distinct umbilical boss, but sometimes several minor less well-defined bosses are seen in the
717 central area. This genetic type (T15) can be linked to the morphospecies *Ammonia falsobeccarii*
718 (Rouvillois, 1974; see Schweizer et al., 2011a).

719 ***4.3.2 Naming cryptic types of Ammonia***

720 Until the partially cryptic genetic types can be conclusively linked to the morphology of type
721 specimens and allocated taxonomic names, they should be named following the system of
722 Hayward et al. (2004) as *Ammonia* sp. T1, *Ammonia* sp. T2A, *Ammonia* sp. T2B, and *Ammonia*
723 sp. T6, to avoid the taxonomic confusion that is prevalent in the literature. This is of course
724 only possible, if genotyping has been carried out. The allocation of either a T-Type or a species
725 name to any cryptic specimens, without the aid of genotyping is not recommended, but if carried
726 out, must be done with care, and any supporting biogeographic and ecological information
727 should be provided.
728
729 Hayward et al. (2004) did apply taxonomic names to a number of the genetic types identified.
730 *Ammonia* sp. T2 has been linked to the taxonomic name *A. aberdoveyensis* Haynes, 1973 (cf.
731 pl. 38, no. 1-2; Holzmann and Pawlowski, 2000; Hayward et al., 2004). Although both T1 and
732 T2 were found at the type locality, T2 was assigned to *A. aberdoveyensis* due to its smaller
733 proloculus in line with the holotype. In this study, we have split T2 into the cryptic types T2A
734 and T2B. Nevertheless, it is T2A that is found at the type locality in the Boreal province, Wales,
735 UK. Although T2B is also found in Wales, it is only found in the Boreal-Lusitanean province
736 further south and appears to be a warmer water species than T2A. It is therefore possible to
737 retain the name *A. aberdoveyensis* for T2A, with the caveat that T2A and T2B cannot be
738 morphologically delineated with confidence. Biogeographical data can be used to assist

739 identification, as it is unlikely that T2B will be found in the North Sea boreal province, whereas
740 T2A has been identified there. However, in warmer waters they can co-occur (Fig. 7; Section
741 4.5.2) and hence care must be taken in taxonomic assignment of specimens in warmer
742 provinces.
743
744 Hayward et al. (2004) also allocated *Ammonia* sp. T6 the taxonomic name *A. aomoriensis*.
745 Hayward's allocation has led to a number of studies using either genotyping (Schweizer et al.,
746 2011b; Lei et al., 2016) or the taxonomic description (Haynert et al., 2012; Nehrke et al., 2013;
747 Langer et al., 2016) for the allocation of their study specimens to the taxon *A. aomoriensis.* We
748 strongly recommend caution in utilising this taxonomic name. The holotype of *Rotalia beccarii*
749 var. *aomoriensis* is from the Pliocene Hamada Formation (Shimokita Peninsula, Aomori
750 Prefecture, Japan), but the taxon is mentioned by Asano (1951) as also occurring in recent
751 material in northern Japan. It is not possible to sequence Pliocene topotype material. Toyofuko
752 et al. (2004) sequenced T6 from modern assemblages of six localities in the nearby area.
753 However, since the oceanographic conditions would have changed markedly since the Pliocene,
754 it is not valid to allocate *A. aomoriensis* to the genetic type T6, despite T6 being found
755 abundantly in the wider region (Lei et al 2016; Supplementary Tables S1 and S5). In addition,
756 we find a number of discrepancies in the taxonomic description of *A. aomoriensis* (Asano,
757 1951) and the morphology of the 50 T6 specimens we have imaged by SEM in our
758 morphometric dataset. The original description states that there are 6-7 chambers in the last
759 whorl whereas T6 has 6-11. It states that the wall is "finely perforate" (a rather broad description
760 that can be assigned to almost any type) and "sutures not limbate". However, several of our T6
761 images show thickened sutures on the spiral/dorsal side. It should be mentioned, however, that
762 the description of *A. aomoriensis* (Asano, 1951) was based on light microscope examination,
763 which may be difficult to compare with SEM observations. We conclude that T6 should not
764 currently be allocated to the taxon *A. aomoriensis* due to morphological discrepancies and a
765 lack of genetic information.
766
767 The taxonomic name *Ammonia tepida* (Cushman, 1926) has been widely used in many studies
768 in the NE Atlantic margins and the Mediterranean Sea, as well as globally. The holotype
769 *Ammonia tepida* from the San Juan Harbour (Puerto Rico), which is recorded in the Cushman
770 Catalogue of 1929, has been re-described by Hayward et al. (2003) and designated as a
771 lectotype. Hayward et al. (2003) concluded (using both morphological observations and DNA
772 sequencing) that the *Ammonia tepida* morphotype has a tropical, equatorial distribution, and
773 that more temperate specimens are of other molecular types and differ in their morphology. We
774 suggest therefore that the taxonomic name *Ammonia tepida* should not be applied to any of the
775 small less-ornate specimens found in in the temperate waters of the NE Atlantic margins.

776 ### *4.4 The distribution of* **Ammonia** *in the NE Atlantic*

777 #### *4.4.1 Northern boundary of* **Ammonia** *morphospecies*

778 In our study, sampling carried out at the northerly sampling sites at Svalbard and Iceland
779 yielded no *Ammonia* specimens. This is in agreement with previous studies which have no
780 recorded observations of *Ammonia* off Iceland (e.g., Nørvang, 1945; Jennings et al., 2004), in
781 the White Sea (Korsun et al., 2014), off the north coast of Norway, in the Tanafjord (70°N;
782 Corner et al., 1996) or slightly further south at Malangenfjord (69°N; Husum and Hald, 2004).
783 The most northerly occurrences of *Ammonia* recorded in the literature were identified in the
784 shallow subtidal areas of the Bergen fjords (Austin and Serjup, 1994; Murray and Alve, 2016).
785 In this study, we also sampled off Bergen, (60°N), but found no *Ammonia* specimens amongst
786 the 271 foraminifera collected there. The most northerly *Ammonia* specimens found in our
787 study were in subtidal samples from the Shetland Islands. No specimens were found here in the
788 intertidal sediments examined, either alive or dead. It is most likely that the near-shore
789 populations of *Ammonia* decline to zero between Bergen (60°N) and Malangenfjord (69°N),
790 and therefore the northern limit of *Ammonia* lies within this region in the present day.
791 Additional sampling in the region would confirm its more exact location. Poole and Vorren
792 (1993) did find *Ammonia* specimens in sediments from the mid-Norwegian shelf (65°- 66°N),
793 but these were fossil foraminifera dating from the Pliocene, a period which was warmer than
794 today (Zachos et al., 2001). We therefore conclude that the most northerly *Ammonia*
795 populations, are currently found at the northern boundary of the Boreal province and in the
796 southern part of the West Norwegian subprovince (Fig. 1).

797 #### *4.4.2 Regional distribution of* **Ammonia** *morphospecies*

798 The distribution of *Ammonia* in the NE Atlantic has been summarised as present from southern
799 Norway to Portugal (Murray, 2006). *Ammonia* has been shown to be prevalent in both the
800 Skagerrak and Kattegat margins (Alve and Murray, 1999; Holzmann and Pawlowski, 2000),
801 down to depths of 70 m (Conradsen, 1993; Conradsen et al., 1994; Bergsten et al., 1996). It has
802 also been observed down to depths of 120 m in the Oslofjord (Risdal, 1964; Alve and Nagy,
803 1990; Alve and Goldstein, 2003). It is perhaps surprising then, that we did not find any subtidal
804 *Ammonia* genotypes in the Skagerrak subprovince, where we collected large numbers of
805 foraminifera (299 specimens adjacent to the Gullmar Fjord (119m); 859 specimens from
806 Oslofjord (22-202 m)). In this study, the first regional sediment samples containing *Ammonia*
807 specimens were collected further south in the Kattegat at Anholt (location 6) in the Boreal
808 province and perhaps unexpectedly in the southern Baltic at Hanö Bay (location 7), where
809 *Ammonia* was thought to be absent (Hermelin, 1987; Murray, 2006: p. 66). However, in 1965
810 Lutze reported its presence in the eastern boundary of the Arkona Basin adjacent to Hanö Bay
811 (location 7) at salinities of 15. In this study, we have found *Ammonia* to be present along the

812 length of the Atlantic European continental margin and into the Mediterranean (Figs. 1, 7 and
813 8), consistent with the literature (e.g., Pawlowski et al., 1995; Holzmann and Pawlowski, 2000;
814 Hayward et al., 2004; de Nooijer et al., 2009; Dissard et al., 2010; Foster et al., 2012; Frontalini
815 et al., 2015; Saad and Wade 2016; LeKieffre et al., 2017; Koho et al., 2018; Tables S1 and S5
816 and references therein). We therefore consider the genus to be ubiquitous in Europe south of
817 60°N.
818
819 There are differences in the abundance and distribution of *Ammonia* between the
820 biogeographical provinces. For example, the continental margins of the North Sea, including
821 the east coast of Scotland, are within the Boreal province, a slightly cooler biome than found
822 on the west coast of Scotland, which is bound by the Boreal-Lusitanean province. This west
823 coast province is characterised by warm waters deriving from the North Atlantic Drift, and is a
824 province of enhanced marine biodiversity, where warm water species appear at comparably
825 high latitudes than in the east (Mitchell et al., 1983; Dinter, 2001; Hiscock and Breckels 2007).
826 This is also observed in our sample set. *Ammonia* was found abundant at latitudes of around
827 56°N at Torry Bay and Cramond (locations 8 and 9) and nearby at South Queensferry (Saad
828 and Wade, 2016) on the east coast of Scotland, and at Dunstaffnage and Loch Sunart (locations
829 4 and 6) on the west coast, at similar latitudes. However further north, differences between the
830 biogeographic provinces were observed. On the east coast in the cool Boreal province only a
831 single specimen was identified at Cromarty (location 2; 57°N), whilst on the west coast, in the
832 warmer Boreal-Lusitanean province, *Ammonia* was commonly found further north at North
833 Uist (location 3; 57°N), where 29 intertidal *Ammonia* were genotyped. *Ammonia* was also
834 observed in Shetland but in subtidal populations only. No intertidal specimens were observed
835 in any of the intertidal mud and seaweeds sampled here. On a northerly transect, therefore, the
836 intertidal *Ammonia* populations decrease prior to the subtidal ones. This makes ecological
837 sense, as the intertidal assemblage would be exposed to greater temperature fluctuations and
838 hence lower temperatures in winter than assemblages in the subtidal zone. The Shetland-
839 Orkney channel also represents a weak eastern boundary between the Boreal-Lusitanean, and
840 the Boreal provinces, and although Shetland sits in the Boreal province, some species here are
841 "southern" species and are not found in other locations in the boreal North Sea (Dinter, 2001).

842 ***4.5 Distribution and ecology of* Ammonia *genetic types in the NE Atlantic***

843 The seven genetic types and subtypes identified in the NE Atlantic margins have both regional
844 and potentially local ecological distinction. This is manifest in differences between the genetic
845 types in their biogeography, depth preferences, or propensity to co-occur. Such information can
846 be used to contribute to our understanding of the possibility of finding a specific *Ammonia*

847 genetic type at a given location, even though they may be morphologically cryptic. The
848 differences in their biogeography are presented in Figs. 7 and 8. The variations in biogeography
849 and habitat and co-occurrences are summarized in Tables 6 and 7.

850 ***4.5.1 Biogeography and ecology of individual genetic types***

851 *Genetic type T1.* – Although rare in our dataset (only eight T1 specimens were identified across
852 four sampling locations (Fig. 7a) in our study) collated data indicate that the T1 genetic type
853 has a broad distribution (Saad and Wade, 2016; Tables S1 and S5). It was found throughout the
854 NE Atlantic margins in a range of biogeographic provinces (the Skagerrak subprovince and the
855 Boreal, Boreal-Lusitanean and Lusitanean-Boreal provinces) and the western Mediterranean
856 Sea (Fig. 7a). T1 has been collected from environments ranging from fully marine intertidal
857 mudflats including estuarine systems to brackish high salt marsh environments (Table 6; Saad
858 and Wade, 2016). It was also identified at subtidal depths (30 m) in low numbers in fjord
859 environments off the west coast of Scotland (Table 1). In our dataset, T1 tended to be the least
860 numerous in mixed *Ammonia* assemblages. However, Saad and Wade (2016) found that T1
861 dominated at two sites on the west coast of the UK within the cool Boreal province of the Irish
862 Sea, but it was not found in the North Sea Boreal province, north of Norfolk (location 13; Figs.1
863 and 7). This, together with its presence further south in the warmer Lusitanean-Boreal province
864 (Holzmann and Pawlowski, 1997) the Mediterranean (Holzmann and Pawlowski, 1997;
865 Pawlowski et al. 1995; 1997) and sub-tropical locations (Hayward et al., 2004) indicate that it
866 tends to prefer relatively warmer waters. It is associated with soft deep muddy sediments and
867 muddy sand sediments (Table 6). T1 is predominantly an intertidal genetic type but has been
868 found subtidally at two sites.
869

870 *Genetic subtype T2A.* – The T2A genetic subtype is a common member of the *Ammonia*
871 assemblage in the Boreal-Lusitanean province and the Boreal provincial regions of the east and
872 south coasts of England (Fig. 7b; Saad and Wade, 2016). However, only a single T2A specimen
873 was found in the *Ammonia* assemblages further north in the western North Sea (location 2, Fig.
874 1; Table 1). Neither was it found in the Boreal provincial coastal waters of the eastern North
875 Sea, in the West Norwegian subprovince off Scandinavia or in the Skagerrak subprovince. This
876 implies that T2A is largely associated with the relatively warmer waters of the Boreal-
877 Lusitanean province and its presence in the southwestern North Sea may be due to the possible
878 encroachment of the Boreal-Lusitanean provincial conditions into the southern North Sea in
879 response to global climate change. Its preference for warmer water is consistent with its
880 presence further south in the warmer Lusitanean-Boreal province (Holzmann and Pawlowski,
881 1997, 2000) and the Mediterranean (Pawlowski et al., 1995; 1997). T2A was collected from
882 soft muddy intertidal sediments and estuarine environments (Table 1). A transect study of the

883 steep shoreline at Dartmouth (location 18; Fig. 9) indicates that T2A is able to survive higher
884 up the shore than the other *Ammonia* genetic types in the intertidal assemblage, suggesting that
885 it has a high tolerance to temperature and salinity extremes. This finding is supported by the
886 study of Saad and Wade (2016), who reported that T2A was found in sandy mud in a high salt
887 marsh habitat, not routinely covered by seawater at every high tide. It was never found
888 subtidally and is therefore an intertidal specialist.
889
890 *Genetic subtype T2B.* – T2B has the most southerly distributed biogeography of all the genetic
891 types identified in the NE Atlantic margins (Fig. 7c). It is the only genetic type (other than the
892 highly restricted T3V) that has not yet been identified in the Boreal province. Its most northerly
893 distribution is around Cork (location 16) and on the Welsh south coast (Saad and Wade 2016),
894 both located in the southern part of the Boreal-Lusitanean province. T2B alone was found at
895 the most southerly sampling location in this study, the Guadiana River (location 22). It has also
896 been found in the Lusitanean-Boreal province and in the Mediterranean Sea (Tables S1 and
897 S5). T2B appears to have a requirement for slightly warmer waters than all the other genetic
898 types identified in the region. Yet, similarly to other partially cryptic types, its habitat
899 preference is still to inhabit intertidal mudflats in estuarine systems composed of soft muddy
900 sediments or hard muddy sand (Table 6; Saad and Wade, 2016).
901
902 *Genetic subtype T3S.* – T3S has the widest biogeographical range of all genetic types identified
903 in this study. It is the most northerly genetic type (Shetland, location 1), and it was identified
904 in nine sampling locations in this study (in the Boreal and Boreal-Lusitanean provinces, the
905 Cool Lusitanean subprovince and the Mediterranean; Fig. 8a). It was also identified in the
906 Skagerrak subprovince (Holzmann and Pawlowski, 2000; Table S1) and the Lusitanean-Boreal
907 province (Ertan et al., 2004). Not only is T3S found in a wide range of biogeographical
908 provinces, it is also found in diverse habitats (Tables 1 and 6) from intertidal seaweeds (location
909 3) and mud (location 2), intertidal estuarine mud (location 18), and subtidal sediments
910 (locations 1, 4, 5, 6, 21 and 22). T3S, therefore, should be regarded as a highly adaptable
911 generalist species in European waters.
912
913 A transect study of the steep shoreline at Dartmouth (location 18), where three genetic types
914 (T1, T2A and T3S) were identified, highlights the differences in their habitat preferences (see
915 section 4.5.2). T3S was found at both low- and mid-shore sites, though numbers were lower in
916 the mid-shore samples. It was completely absent from the upper shore. The drop off in numbers
917 up-shore fits with our understanding that T3S is both an intertidal and subtidal genetic type,
918 also found in the deepest sites where *Ammonia* was identified in this study.
919

920 *Genetic subtype T3V.* – Particularly interesting is that this genetic subtype is highly localised
921 to the region of Vendée, on the French Atlantic coast (Fig. 8b; Pawlowski et al., 1995; Ertan et
922 al., 2004; Hayward et al., 2004; this study). We collected T3V from intertidal seaweeds on Ile
923 d'Yeu (location 19) off the Vendée coast. All specimens reported in the literature were from
924 intertidal habitats, but whether from sediments or seaweeds is unknown.
925
926 *Genetic type T6.* – This is the only genetic type that has been widely reported in the Boreal
927 province of the North Sea (Fig. 7d), where *Ammonia* is considered ubiquitous. T6 may therefore
928 account for the majority of the *Ammonia* specimens sampled from this region. It is also found
929 in the Boreal province of the Irish Sea (Saad and Wade 2016), and to a lesser extent in the
930 Boreal-Lusitanean province, where it is found on the Welsh south coast (locations 14 and 17)
931 bordering the Boreal province. It was not found further south in the Boreal-Luistanean province
932 at either Cork (location 16) or Dartmouth (location 18) in this study. We did, however, identify
933 two T6 specimens further south at Baie de l'Aiguillon in the Lusitanean-Boreal province. We
934 did not find T6 further south in the Portuguese margin (location 22) or the Rhône prodelta
935 (location 21) in the Mediterranean, but these fully marine subtidal habitats are not preferred by
936 T6. However, despite a range of intertidal sampling in the Gulf of Lions (at Camargue, Le
937 Boucanet and Banyuls-sur-Mer; see Tables S1 and S5) and in the Adriatic Sea (Trieste, Lagoon
938 of Venice; Tables S1 and S5) this genetic type has not been reported there to date.
939
940 We have found T6 widely in brackish environments on the intertidal and estuarine shores within
941 our study area and at one subtidal site of low salinity (location 7). This supports the finding of
942 Schweizer et al. (2011b), who identified T6 subtidally (between 4-14 m) in the Kiel Fjord, in a
943 lens of low salinity Baltic seawater. Such salinities may be more akin to the intertidal and
944 estuarine environments, in which T6 has thus far has been found. Contrary to this, T6 was also
945 found in the saline Grevelingen Lake (location 15) in The Netherlands. The lake was part of
946 the Rhine River delta prior to being dammed, with a later edition of a sluice gate resulting in
947 salinities of 29-32 (Hagens et al., 2015). T6 may be an invading species via the sluice, as it is
948 widely distributed along the coast here. Cores from Grevelingen show that prior to damming,
949 the *Ammonia* specimens present had smaller pores, suggesting the presence of T2A rather than
950 T6. After the closure of the lake, there was a shift to specimens with larger pores, suggesting
951 an invasion of the lake by T6 via the sluice gate (Petersen et al., 2016). Its presence at these
952 marine salinities, would indicate that it is a euryhaline species, despite a probable preference
953 for brackish environments. Saad and Wade (2016) found *Ammonia* T6 around the UK in 14 of
954 19 sites sampled, and all but one of these 14 sites were described as brackish. The final sampling
955 site, where T6 was identified however (Barrow-in-Furness; on the English west coast in the
956 Boreal province), was described by these authors as fully marine. However, no salinity

957 measurements were reported for any of the sampling sites and certainly, the majority of sites
958 where T6 has been found are intertidal and estuarine mudflats, which will experience
959 fluctuating salinities.
960
961 *Genetic type T15.* – T15 is relatively rare in our dataset. However, it was identified along the
962 NE Atlantic margins where *Ammonia* is found at subtidal locations in the Boreal and Boreal-
963 Lusitanean provinces, the Cool-Lusitanean subprovince and the Mediterranean (Fig. 8c). The
964 only other molecular data available for this genetic type identified T15 in the Mediterranean
965 Sea (Rhône prodelta) and the Bay of Biscay in accordance with previous morphologically based
966 studies which also found this type in the Adriatic Sea (Schweizer et al., 2011a and references
967 therein). Subtidal sampling off Shetland yielded no T15 specimens, whilst T3S was identified
968 here. This may be either due to the presence of cooler waters, or the requirement for more
969 extensive regional sampling. It is important to note that T15 is a fully marine species, restricted
970 to subtidal muddy organic matter rich habitats.

971 ***4.5.2 Co-occurrence of* Ammonia *genetic types***

972 It is of great importance for accurate data interpretation, to know whether more than one
973 *Ammonia* genetic type is present, at any given sampling site. Of the 22 locations in this study,
974 13 locations contained only one genetic type, six locations contained two genetic types and
975 three locations contained three genetic types. Their degree of co-occurrence is documented in
976 Tables 6 and 7. Of significance is the fact that the smaller, less ornate cryptic genetic types T1,
977 T2A, T2B and T6 co-occur in a variety of combinations (T1+T2A, T2A+T6, T1+T2B). In
978 addition, T3S also co-occurs with T1 and T2A intertidally, as well as T1 and T15 subtidally. In
979 agreement with our data from Cork, GenBank sequences (Supplementary Table S5) also place
980 genetic types T1 and T2B together in Trieste in Italy, and with T2A in the Gulf of Lions
981 (Camargue, French Mediterranean coast) and in the Lagoon of Venice, Italy (Holzmann et al.,
982 1996; Pawlowski et al., 1997; Holzmann and Pawlowski, 2000). It is noteworthy that, despite
983 T3S and T6 being relatively abundant and found at nine separate locations each, they were
984 never identified together (Table 7). This is most likely due to their differing ecological
985 preferences, but this requires further sampling for confirmation.
986
987 The presence of different combinations of genetic types at different locations along a single
988 shore transect at Dartmouth highlights the importance of clarifying the exact location of
989 sampling on the intertidal shore (Fig. 9). For example, where T2A has been identified alone
990 (e.g., locations 2 and 11, this study; Lymington: Saad and Wade, 2016), it is possible that only
991 the upper shore was sampled, and that sampling the lower shore might reveal co-occurrence

992 with other genetic types. We therefore recommend sampling at different heights on the
993 shoreline, or to record the height at which samples are taken.
994
995 Of particular interest is that T6 very rarely co-occurred with the other genetic types in this study.
996 Indeed, we identified only one example (a single specimen of T2A co-occurring with T6 at
997 Norfolk; location 13; Table 7). Also, of the 14 sampling locations in the UK investigated by
998 Saad and Wade (2016) where T6 was identified, T6 inhabited eleven sites alone and co-
999 occurred with a second genetic type at only three sites, all in the Boreal province. In continental
1000 Europe, T6 was found alone in the Boreal province on the German coastline at Wilhelmshaven
1001 (Holzmann and Pawlowski, 2000), Crildumersiel (Langer and Leppig, 2000) and Amrum
1002 (Ertan et al., 2004) and on the coast of The Netherlands at Den Oever (Schweizer et al., 2011b).
1003 However, it was also found with T1 in The Netherlands at a single location, Mok Baai,
1004 (Holzmann and Pawlowski, 2000). In total, T6 has been reported at 29 locations in the NE
1005 Atlantic margins, but only co-occurred at five. This indicates that it may be a highly robust
1006 genetic type, able to out-compete others when salinity and other conditions favour it.

1007 ***4.6 Global biogeographical patterns of the NE Atlantic* Ammonia *genetic types***

1008 Outside Europe, T1 genetic types were identified in Australia, New Zealand, New Caledonia,
1009 Chile, Cuba, and the USA east coast (Supplementary Tables S1 and S5), demonstrating the
1010 cosmopolitan nature of this genetic type, despite the low numbers we observed in the NE
1011 Atlantic margins. On the other hand, a single GenBank LSU sequence originating from Cape
1012 Cod in the USA (Supplementary Table S5) identified T2A as potentially confined to the
1013 Atlantic, as it has yet to be identified in the Pacific or Indian Oceans. T3S has not been reported
1014 outside Europe (Hayward et al., 2004). However, in this study, T3S was found subtidally at six
1015 out of the nine sampling locations where it was identified. If global sampling was largely
1016 confined to intertidal margins, it may have been missed, as in the intertidal study around the
1017 British Isles by Saad and Wade, (2016). However, several potential endemic *Ammonia* genetic
1018 types have been identified in other regions globally (Hayward et al, 2004), suggesting that T3S
1019 could equally be endemic to the NE Atlantic. This possibility is confirmed by the extreme
1020 endemism exhibited by its sister genetic type T3V, which appears isolated within a small coastal
1021 region of France in the NE Atlantic. The genetic type T6 has only been found in Europe, Japan
1022 and China to date (Holzmann and Pawlowski, 2000; Hayward et al., 2004; Schweizer et al.,
1023 2011b) (Tables S1 and S5; Fig.7c), and this disjunct distribution may indicate a possible exotic
1024 species (discussed below). T2B, originally designated as part of the T2 cluster by Hayward et
1025 al. (2004; Fig. 3), has yet to be identified outside European waters (Hayward et al., 2004) again
1026 implying that it may be endemic to NE Atlantic margins and the Mediterranean. Finally, T15

1027 has not been documented from other regions, but this may be due to its subtidal nature and the
1028 predominance of intertidal sampling globally as mentioned above.
1029
1030 There are a number of genetic types found in other regions that have not been identified in the
1031 NE Atlantic margins. For example, genetic types T7 and T9 are found on the east coast of the
1032 USA in temperate waters, but unlike T1 and T2A that are also found in the NE Atlantic, they
1033 are not transatlantic genetic types. The reasons for the differences in the biogeography of these
1034 genetic types is not yet clear, but may be a function of their ecology and the NE Atlantic
1035 circulation. T11 is found in the Caribbean and Cuba but has not been found further north on the
1036 USA coastline. T11 has not been found in the NE Atlantic margins and is most likely a warmer
1037 water specialist. This is in direct comparison to the ubiquitous T1, which although found in
1038 warm Cuban waters, is also a transatlantic genetic type able to tolerate wide temperature
1039 gradients.

1040 ***4.7 Potential expatriation of T6***

1041 The disjunct distribution of T6 in the North Sea, China and Japan observed by Hayward et al
1042 (2004) led to the hypothesis that it originally came from Asia through ship ballast water to the
1043 North Sea (Pawlowski and Holzmann, 2008). Evidence for this came from the congruent
1044 distribution in Asia of *Ammonia* sp. T6 with the decapod *Eriocheir sinensis,* which was
1045 introduced to the Wadden Sea at the end of the 19th century via shipping (Nehring and Leuchs,
1046 2000). In the present study, and that of Saad and Wade (2016), it has been shown that the
1047 distribution of T6 is far broader in Europe than previously recorded (Fig.7d). There are two
1048 possible inferences from this. Firstly, increased sampling of its favoured estuarine mudflat
1049 habitats might reveal a non-disjunct distribution, with a more global dispersal for T6 than
1050 currently recognised. For example, T6 has been found far up in to the Forth River system at
1051 both Torry Bay (location 8) and Cramond (location 9). Secondly, the observed wider
1052 distribution in Europe might infer that invasion via ballast has led to extremely rapid
1053 colonisation of a wide area (including the UK west coast; Saad and Wade, 2016), due to its
1054 adaptable euryhaline nature. T6 may be an aggressive invasive species, able to outcompete
1055 indigenous genetic types. Down-core sampling to check the presence of T6 in Europe in the
1056 past decades or centuries would be of benefit. However, the morphological identification of T6
1057 and its discrimination from other genetic types (T1, T2A and T2B) would be required (see
1058 Richirt et al., in press).
1059
1060 Core data from the outer Kiel Fjord demonstrates the late arrival (2000) of *Ammonia* to the
1061 area. This coincided with a decrease in salinity that favoured invasion of the fjord by *Ammonia*

1062 and excluded the strong-halocline adapted *Ammotium cassis* that previously made up to 90%
1063 of the foraminiferal abundance (Polovodova et al., 2009). Genetic characterisation of the
1064 *Ammonia* genetic types in the Kiel Fjord identified them as T6 (Schweizer et al., 2011b).
1065 Although *Ammonia* was thought to be absent from the Baltic Sea under the present salinity
1066 conditions (Hermelin, 1987; Murray, 2006: p. 66), we have demonstrated the presence of T6
1067 also in Hanö Bay (location 7), with a population that could have been seeded by propagules
1068 from the Kiel Fjord, and the Kattegat and Skagerrak Seas (see Fig. 7d). The question remains
1069 as to the original source of *Ammonia* sp. T6 to the area. It is not known whether it is a globally
1070 distributed genetic type, that has slowly moved into the Kattegat and Baltic Seas as conditions
1071 have become more favourable to it, or if it is indigenous to China and Japan, transported in
1072 ballast water to the North Sea area and rapidly colonising the region, or vice versa. Only further
1073 global sampling of the brackish environments that it prefers will provide clues to its full
1074 biogeography.

1075 **5 Summary and conclusions**

1076 This study represents the first major genetic, biogeographic and morphometric investigation
1077 carried out on *Ammonia* specimens within the NE Atlantic margins. Here, *Ammonia* comprises
1078 seven genetic types and subtypes (T1, T2A, T2B, T3S, T3V, T6 and T15). Phylogenetic
1079 analyses were unable to resolve the relationships between the subtypes T2A and T2B or T3S
1080 and T3V and a focussed genetic survey of their intra-individual SSU variants is required to
1081 establish their genetic distinction and biogeography. The nomenclature for classifying the
1082 degree of genetic separation within and between benthic foraminiferal morphospecies and
1083 genera such as *Ammonia* are in serious need of stability and clarification. Morard et al., (2016),
1084 have proposed a nomenclature for use in planktonic foraminifera that can be applied to
1085 prescribed levels of divergence. We would argue for its adoption for benthic foraminifera, as it
1086 would provide a framework for characterising and the naming the different levels of genetic
1087 divergence we observe.
1088 This study has demonstrated that ecological niches can be used to help discriminate between
1089 *Ammonia* genetic types within the NE Atlantic margins. Subtidal *Ammonia* specimens will
1090 either be the morphologically distinguishable genetic types T3S (*A. batava*) or T15 (*A.*
1091 *falsobeccarii*). In fully marine subtidal regions, T1 may also be present, which is
1092 distinguishable from both T3S and T15. However, in more brackish subtidal waters, T1, T3S
1093 and T15 will not be present, and *Ammonia* specimens here are likely to be T6. Intertidal
1094 specimens are more difficult to delineate, particularly since co-occurrence of two to three types
1095 is common. However, the proportionate composition of upper slope genetic types differs from
1096 that of the lower slope ones, and this knowledge together with the biogeographical distribution

1097 of the different types contributes significant information towards the enhancement of
1098 (palaeo)ecological regional studies. This demonstrates the importance and value of identifying
1099 *Ammonia* at the biological species level instead of lumping them as cosmopolitan morphotypes,
1100 which provides limited environmental information.
1101

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1622 **Figures**

1623 Fig. 1. Map of the NE Atlantic showing sampling locations in this study. Open circles (o) are
1624 locations where *Ammonia* was absent, and closed circles (●) are locations where *Ammonia* was
1625 successfully sequenced (numbered north to south, see Table 1). The map also shows the
1626 biogeographic classification of the benthic and neritopelagic regions of the shelf and upper
1627 continental slope (Dinter, 2001: Fig. 105).
1628
1629 Fig. 2. SEM image plate showing representative specimens typical of each *Ammonia* genetic
1630 type with umbilical and spiral sides. The apertural side is also presented for some individuals.
1631 Scale bar 100 μm.
1632
1633 Fig. 3. Molecular phylogeny of *Ammonia* based on partial SSU sequences inferred using the
1634 BioNJ method with the K2P model. The tree is unrooted and support values for BioNJ/ML-
1635 HKY+Γ/BA are indicated at the main nodes. Individual sequences are labelled with the SSU
1636 genetic types (S) where known and/or T-types (Hayward et al., 2004).
1637
1638 Fig. 4. UPGMA cluster dendrogram based on the morphological characteristics (Table 3) of the
1639 seven *Ammonia* genetic types across the NE Atlantic margins (n=158).
1640
1641 Fig. 5 Primary PCO analysis of the morphometric data of the seven distinct genetic types found
1642 in the NE Atlantic margins. Each group is bounded by a convex hull. The first two principle
1643 coordinates account for 35.6% of the total variation.
1644
1645 Fig.6. Secondary PCO analysis of the *Ammonia* morphometric data, excluding T3S and T3V,
1646 which were separated in the primary PCO analysis (Fig. 5). Each of the genetic types is bounded
1647 by a convex hull. The two principle components account for 28.8% of the total variation.
1648
1649 Fig. 7. Biogeographical distribution maps for the small less ornate, morphometrically
1650 overlapping genetic types; T1, T2A, T2B and T6. Biogeographic provinces where genetic types
1651 are located are shaded grey. Closed circles (●) represent specimens genetically identified in this
1652 study; open triangles (Δ) represent SSU sequences already in GenBank; and open squares (□)
1653 represent LSU sequences already in GenBank or specimens identified by restriction fragment
1654 length polymorphism (Denmark, T6 only).
1655
1656 Fig. 8. Biogeographical distribution maps of the morphologically identifiable genetic types
1657 T3S, T3V and T15. Biogeographic provinces where genetic types are located are shaded grey.

1658 Closed circles (●) represent specimens genetically identified in this study; open triangles (Δ)
1659 represent SSU sequences already in GenBank; and open squares (□) represent LSU sequences
1660 already in GenBank.
1661
1662 Fig. 9. Cross section of a shore transect taken at Dartmouth (UK). Pie charts show proportions
1663 of genetic types identified in each of the upper-, mid- and lower-shore samples. Numbers in
1664 brackets are the number of individuals genetically characterised. Lower-shore samples were
1665 taken at extreme low tide within four days of the low spring tide event. Upper-shore samples
1666 were collected from the marine sediment below the transition from sediment to grass. Mid-
1667 shore samples were taken approximately midway between the two, but using the mid-shore
1668 indicator seaweed, *Fucus vesiculosus,* as a guide.

1669 **Tables**

1670 Table 1. Description of sampling locations and the *Ammonia* genetic types identified.
1671
1672 Table 2. *Ammonia* SSU sequences used for phylogenetic analyses (Fig. 3) including sequences
1673 from this study and those previously deposited in GenBank. References are either where the
1674 sequences were first published or direct submissions to GenBank (DS). Accession numbers are
1675 shown with previously published sequences in italic and new ones (this study) in bold.
1676
1677 Table 3. Test characteristics measured or assessed from the umbilical and spiral SEM images
1678 of the *Ammonia* specimens. These measured morphological characteristics have been derived
1679 from Hayward et al. (2004) with some minor modifications. The qualitative five-point
1680 assessment utilised in this study includes: 1- None, 2- Very weak, 3- Weak, 4- Medium, and 5-
1681 Strong. The three-point scale utilised here includes: 1- Absent, 2- Moderately developed, 3-
1682 Strongly developed. Chamber N is equivalent to the final chamber, whilst N1 is the penultimate
1683 chamber etc.
1684
1685 Table 4. Conversion of SSU genetic types (S) from this study into the established T-type
1686 nomenclature originally based on the LSU (Holzmann and Pawlowski 2000; Hayward et al.,
1687 2004).
1688
1689 Table 5. Confusion matrix of the number of *Ammonia* specimens correctly and incorrectly
1690 classified into each genetic type in the Discriminant Function Analysis and cross validation
1691 procedure. Percentage of correctly classified individuals is also reported for each genetic type.
1692 T3V was not included in the DFA due to the small number of images available for analysis.

1693

1694 Table 6. Description of the biogeographical range, habitat and co-occurrence of the seven
1695 genetic types and subtypes identified in this study. The biogeographical ranges described
1696 include specimens whose sequences have been previously deposited in GenBank by others
1697 (Tables S1 and S5), and are as shown on maps Figs. 7 and 8. Biogeographic provinces are based
1698 on the OSPAR Maritime Areas (Dinter, 2001). Habitat descriptions and co-occurrences are
1699 based on this study and Saad, and Wade (2016).

1700

1701 Table 7. Number of specimens genetically characterised from each of the 22 sampling
1702 locations.

1703

1704 **Supplementary figures**

1705 Fig. S1. Molecular phylogeny of *Ammonia* based on partial SSU sequences inferred using the
1706 ML method with the HKY+Γ model. The tree is unrooted and bootstrap values (1000 replicates)
1707 are indicated at the nodes.

1708

1709 Fig. S2. Molecular phylogeny of *Ammonia* based on partial SSU sequences inferred using the
1710 ML method with the GTR+Γ model. The tree is unrooted and bootstrap values (1000 replicates)
1711 are indicated at the nodes.

1712

1713 Fig. S3. Molecular phylogeny of *Ammonia* based on partial SSU sequences inferred using the
1714 BA method with the mixed model. The tree is unrooted and posterior probabilities are indicated
1715 at the nodes.

1716 **Supplementary tables**

1717 Table S1. *Ammonia* partial SSU sequences retrieved from the GenBank database (April 2015)
1718 used in the SSU alignment. References are either the articles where the sequences were first
1719 published or direct submissions (DS).

1720

1721 Table S2. The range of measurements of each morphological test characteristic for each genetic
1722 type. The qualitative five-point assessment utilised in this study (Table 3) includes: 1- None, 2-
1723 Very weak, 3- Weak, 4- Medium, and 5- Strong. The three-point scale utilised includes: 1-
1724 Absent, 2- Moderately developed, 3- Strongly developed.

1725

1726 Table S3. Number of *Ammonia* specimens genetically characterised by sequencing or screening
1727 and new SSU sequences submitted to GenBank (this study). SSU sequences already published
1728 in GenBank for each genetic type (July 2018) are also shown. Genetic types in bold are those
1729 represented in NE Atlantic margins.
1730
1731 Table S4: Link between SSU and LSU genetic types sequenced in the same individuals with
1732 GenBank accession numbers corresponding to each gene. Accession numbers in italics are
1733 previously published, those in bold are this study.
1734
1735 Table S5. *Ammonia* partial LSU sequences retrieved from the GenBank database (August 2015)
1736 with additional sequences from Saad and Wade (2016). References are either the articles where
1737 the sequences were first published or direct submissions (DS).

1738 **Supplementary information**

1739 Supplementary Data 1. Complete set of morphometric measurements for each *Ammonia*
1740 specimen morphometrically analysed.
1741
1742 Supplementary Data 2. Alignment of LSU sequences showing variability between genetic
1743 subtypes T2A and T2B, and T3S and T3V.
1744
1745 Supplementary Data 3. Alignment of SSU sequences showing minor variation between genetic
1746 subtypes T3S and T3V.