

A note on the phylogenetic position of *Entzia macrescens* (Brady)

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Abstract

We extracted and amplified a fragment of small subunit ribosomal DNA (SSU rDNA) from three *Entzia macrescens* specimens collected in Turda, Romania. The three sequences build a monophyletic group together with a sequence of *E. macrescens* obtained from Dovey Estuary, Wales, and branch as sister to five sequences of *Entzia* sp. specimens from Camargue, France. The two groups of *E. macrescens* and *Entzia* sp. build a strongly supported monophyletic clade with *Haplophragmoides* spp. and *Arenoparella mexicana* at its base. Calculation of pairwise distances shows that observed interindividual and intraindividual distances in *E. macrescens* and *Entzia* sp. are similar and lower than interspecific distances which indicates that the population from Camargue might belong to a different, cryptic species.

Migratory birds might be responsible for the transport of *E. macrescens* to the land locked salt marshes of Romania. The sequences of *E. macrescens* from Dovey Estuary and Turda are almost identical, which would confirm the former hypothesis as flyways extend from Great Britain to the Southeast via Eilat to Africa that cross Romania. There is no migratory flyway that crosses the European continent from the West (France) to the Southeast, which possibly limits the aerial distribution of *Entzia* sp. found in Camargue.

INTRODUCTION

Entzia macrescens (Brady, 1870) is a planispirally enrolled multichambered foraminifera with an agglutinated test that is ranked in the superfamily Trochamminacea. Traditional classification of trochamminid foraminifera is based on test shape, wall structure and form and placement of apertures (Loeblich & Tappan, 1988, Dorst & Schönfeld, 2015). However, the limited number of morphological features used for distinction makes it sometimes difficult to identify species in trochamminid Foraminifera (Pawlowski *et al.*, 1995).

Entzia macrescens specimens that were sampled from a salt marsh in Transylvania have been at the base of a redescription of the genus by Filipescu & Kaminski (2011). In the current phylogenetic study we show that *E. macrescens* specimens from Romania and Great Britain build a monophyletic group that branches as sister to *Entzia* sp. from Camargue. The genus *Entzia* probably contains cryptic species that may not be distinguishable using morphological features only.

MATERIALS AND METHODS

Sampling

Live specimens of *Entzia macrescens* and *Entzia* sp. were

isolated from sediment samples by use of a binocular microscope. Isolated specimens of *E. macrescens* from Romania were subsequently transferred to a micropaleontological slide, dried at ambient temperature and sent to Geneva by mail for further analysis.

Several specimens from each sampling locality were preserved on micropaleontological slides for morphological studies and SEM photographs of *E. macrescens* from Dovey Estuary and *Entzia* sp. from Camargue are presented on Plate 1.

DNA Extraction, Amplification, Cloning and Sequencing

DNA from four specimens of *Entzia macrescens* and two specimens of *Entzia* sp. was extracted using guanidine lysis buffer (Pawlowski, 2000). DNA isolate numbers and collection sites are given in Table 1. PCR amplifications of the 5'end fragment of the SSU rDNA were performed using primer pairs s14F3-sB and 14F1-sB as indicated in Table 2.

The amplified PCR products were purified using High pure PCR Purification Kit (Roche Diagnostics) cloned with the TOPO TA Cloning Kit (Invitrogen) following the manufacturer's instructions and transformed into competent *E. coli*. Sequencing reactions were performed using the BigDye

Table 1: Isolate numbers and sampling locations of sequenced specimens

Isolate number	Species	Sampling location
229	<i>Arenoparrella mexicana</i>	USA, Sapelo Island
FF138	<i>Cribrostomoides</i> sp.	New Zealand
2720	<i>Cribrostomoides crassimargo</i>	Norway, Svalbard
139	<i>Eggerelloides scaber</i>	France, Bretagne
504	<i>Entzia macrescens</i>	France, Camargue
505	<i>Entzia macrescens</i>	France, Camargue
418	<i>Entzia macrescens</i>	GBR; Dovey Estuary
13530	<i>Entzia macrescens</i>	Romania, Turda
13533	<i>Entzia macrescens</i>	Romania, Turda
13534	<i>Entzia macrescens</i>	Romania, Turda
1241	<i>Haplophragmoides</i> sp.	Canada, Halifax
417	<i>Haplophragmoides wilberti</i>	GBR; Dovey Estuary
E17	<i>Reophax scorpiurus</i>	Norway, Svalbard
1879	<i>Reophax</i> sp.	Antarctica
2646	<i>Spiroplectammina</i> sp.	Norway, Svalbard
17015	<i>Textularia agglutinans</i>	Israel, Eilat
13633	<i>Textularia gramen</i>	Faroe Islands

Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analysed on a 3130XL Genetic Analyzer (Applied Biosystems).

Phylogenetic Analysis

The obtained sequences were added to an existing database using the Muscle automatic alignment option as implemented in Seaview vs. 4.3.3. (Gouy *et al.*, 2010). The alignment of SSU rDNA gene sequences contained 23 taxa and 1142 sites used for the analysis.

Based on automatic model selection as implemented in ATGC (Guindon *et al.*, 2010), a GTR model of evolutionary changes was selected for the analysis. A phylogenetic tree was constructed using maximum likelihood (PhyML) method using PhyML 3.0 as implemented in ATGC. Bootstrap values are based on 100 replicates.

Pairwise comparison of distances has been calculated for the 23 taxa present in the tree using MEGA5 (Tamura *et al.*, 2011), the results are presented in Table 3.

RESULTS AND DISCUSSION

Entzia macrescens and *Entzia* sp. build a monophyletic highly supported (100% BV) clade (Fig. 1). A second clade containing *Haplophragmoides* sp., *H. wilberti* and *Arenoparrella mexicana* branches at the base of *Entzia* spp. The two clades are supported by a high bootstrap value (97% BV). Another clade containing *Textularia agglutinans*, *Textularia gramen*, *Trochammina hadai*, *Eggerelloides scaber* and *Spiroplectammina* sp. (82% BV) branches at the base of the former two clades, the branching is supported by 82% BV. *Cribrostomoides* sp. and *C. crassimargo* branch at the base of the former clades but the branching is not supported. *Reophax scorpiurus* and *Reophax* sp. (100% BV) were chosen as outgroup.

It is interesting to note that the strongly supported sister clades of *Entzia* spp, *Haplophragmoides* spp. and *A. mexicana* consist of planispirally enrolled foraminifera. The genus *Entzia* contains two phylotypes, one of them, *E. macrescens* has been redescribed by Filipescu & Kaminski

Table 2: PCR primers used for amplification and reamplification of SSU rRNA genes

Name	Sequence	forward	reverse	Amplification	Reamplification	Target
s14F3	acgcamgtgtgaaacttg	x		x		SSU rDNA
sB	tgatccttctgcaggttcacctac		x	x	x	SSU rDNA
s14F1	aagggcaccacaagaacgc	x			x	SSU rDNA

Table 3: Estimates of Evolutionary Divergence between Sequences. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model. The analysis involved 23 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1142 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1 229_Arenoparrella mexicana																						
2 FF138_Cribrostomoides sp.	0.188																					
3 2720_Cribrostomoides crassimargo		0.025																				
4 1241_29_Haplophragmoides sp.	0.154	0.170	0.168																			
5 1241_30_Haplophragmoides sp.	0.148	0.166	0.164	0.005																		
6 1241_31_Haplophragmoides sp.	0.149	0.165	0.163	0.004	0.003																	
7 504_21_Entzia sp._Camargue	0.176	0.152	0.149	0.113	0.110	0.110																
8 504_22_Entzia sp._Camargue	0.173	0.153	0.151	0.108	0.105	0.105	0.011															
9 505_26_Entzia sp._Camargue	0.176	0.156	0.153	0.110	0.106	0.106	0.014	0.009														
10 505_27_Entzia sp._Camargue	0.176	0.151	0.151	0.109	0.106	0.106	0.013	0.006	0.011													
11 505_28_Entzia sp._Camargue	0.175	0.151	0.148	0.109	0.106	0.106	0.010	0.005	0.008	0.007												
12 418_Entzia macrescens_Dovey Estuary	0.165	0.151	0.145	0.111	0.107	0.107	0.036	0.031	0.035	0.033	0.032											
13 13530_Entzia macrescens_Romania	0.161	0.150	0.145	0.108	0.105	0.105	0.033	0.028	0.032	0.031	0.029	0.004										
14 13533_Entzia macrescens_Romania	0.163	0.153	0.149	0.106	0.103	0.103	0.032	0.028	0.031	0.030	0.028	0.008	0.004									
15 13534_Entzia macrescens_Romania	0.164	0.152	0.147	0.111	0.107	0.107	0.033	0.027	0.032	0.030	0.029	0.006	0.002	0.002								
16 2646_Spiroplectammina sp.	0.185	0.155	0.150	0.156	0.152	0.150	0.172	0.167	0.172	0.168	0.169	0.161	0.161	0.163	0.162							
17 95_Trochammina hadai	0.178	0.136	0.137	0.146	0.143	0.142	0.153	0.148	0.152	0.147	0.149	0.146	0.145	0.145	0.146	0.118						
18 13633_Textularia gramen	0.186	0.136	0.140	0.155	0.153	0.152	0.147	0.149	0.152	0.148	0.149	0.142	0.140	0.142	0.141	0.118	0.089					
19 17015_Textularia agglutinans	0.203	0.131	0.134	0.168	0.162	0.162	0.145	0.147	0.147	0.149	0.148	0.141	0.139	0.143	0.140	0.117	0.092					
20 139_Eggerelloides scaber	0.169	0.151	0.150	0.163	0.160	0.160	0.164	0.163	0.167	0.164	0.164	0.161	0.159	0.154	0.161	0.143	0.132	0.122				
21 E17_Reophax scoparius	0.183	0.121	0.116	0.135	0.133	0.132	0.134	0.133	0.137	0.133	0.132	0.132	0.130	0.133	0.132	0.124	0.120	0.132	0.148			
22 1879_Reophax sp.	0.206	0.154	0.149	0.163	0.159	0.158	0.161	0.162	0.165	0.164	0.162	0.159	0.156	0.154	0.157	0.160	0.150	0.156	0.141	0.168		
23 417_Haplophragmoides wilberti	0.159	0.192	0.192	0.107	0.104	0.104	0.121	0.122	0.120	0.121	0.120	0.119	0.115	0.116	0.117	0.160	0.160	0.156	0.171	0.155	0.120	
Intraindividual distance																						
Interindividual distance																						
Interspecific distance																						

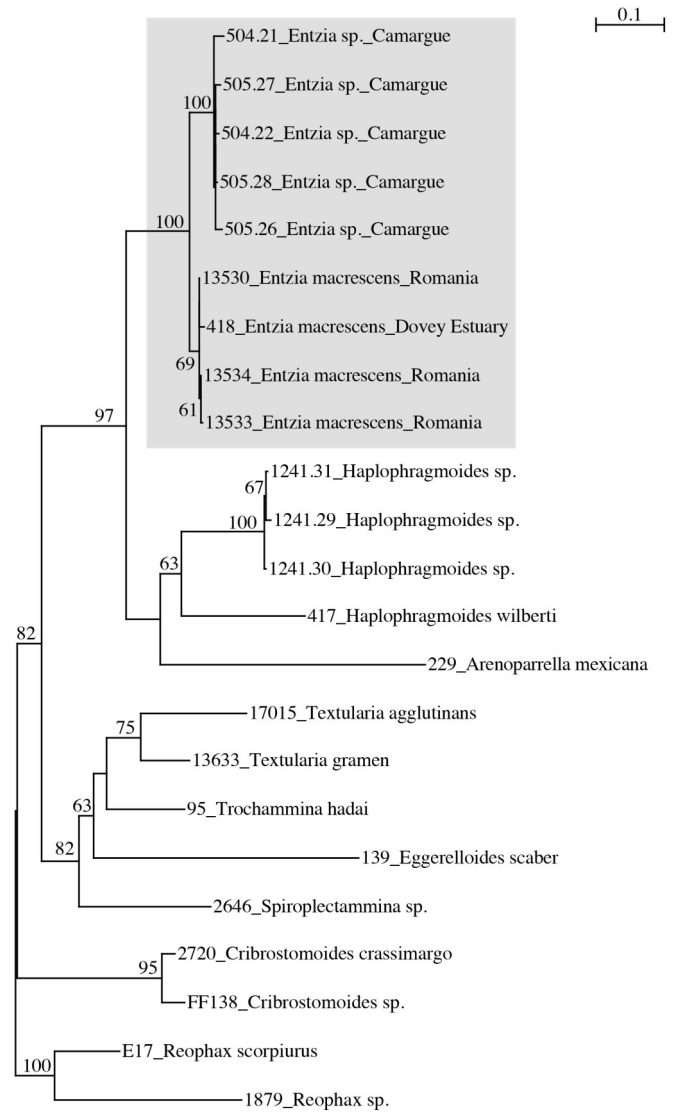


Fig. 1: PhyML phylogenetic tree showing evolutionary relationships of textulariid foraminifera inferred from 23 ribosomal DNA sequences. Numbers at nodes indicate bootstrap values. The tree was rooted with *Reophax scoparius* and *Reophax sp.*

(2011). The other one is represented by five sequences derived from two specimens sampled in Camargue. Intraindividual distances in *Entzia sp.* mount from 0.007 to 0.011 (Table 3) while interindividual distances mount from 0.005 to 0.014. In *E. macrescens* interindividual distances mount from 0.002 to 0.008. Intra- and interindividual distances are thus similar in *Entzia* spp. while interspecific distances mount from 0.027 to 0.036.

In *Haplophragmoides sp.* intraindividual distances mount from 0.003 to 0.005 while interspecific distances between the former species and *H. wilberti* mount from 0.104 to 0.107. Interspecific distances in *Cribrostomoides* spp. and *Textularia* spp. mount to 0.025 and 0.092 respectively. In-

traindividual distances are thus similar in *Entzia* sp. and *Haplophragmoides* sp. while interspecific distances are similar in *Entzia* spp. and *Cribr stomoides* spp.

Entzia specimens from Camargue probably represent a different, cryptic species that is morphologically similar to *E. macrescens* (Plate 1). Further investigations will be necessary to clarify this question.

Entzia macrescens has been sampled from land locked salt marshes in Transylvania. Migratory birds offer an explanation for the possible transport of *E. macrescens* specimens. It is estimated that more than one quarter of all European breeding birds are migratory birds and that approximately 2.1 billion birds travel between Europe and Africa twice each year (Hahn *et al.*, 2008). There are several main routes of bird migration from Europe and Asia to Africa and one of these flyways leads from Great Britain through the European continent to Turkey, Eilat (Israel) and Africa (Ramachandra *et al.*, 2011). This flyway crosses Romania and *E. macrescens* might be distributed in the course of yearly migrations. The hypothesis of bird migration is supported by the fact that the sequences of *E. macrescens* from Dovey Estuary and Romania are almost identical.

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There are no bird migratory routes that traverse the European continent from the west to the east, which might explain the fact why *E. macrescens* is found in Romania but not *Entzia* sp.

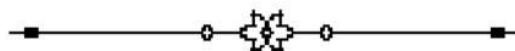
CONCLUSIONS

Our phylogenetic results show that *Entzia macrescens* and *Entzia* sp. build strongly supported monophyletic groups that cluster together. Almost identical sequences of *E. macrescens* have been obtained from Turda, Romania and Dovey Estuary, Great Britain. *Entzia macrescens* specimens might be distributed by migrating birds.

A second phylotype of *Entzia* sp. is present in Camargue and probably presents a new, cryptic species.

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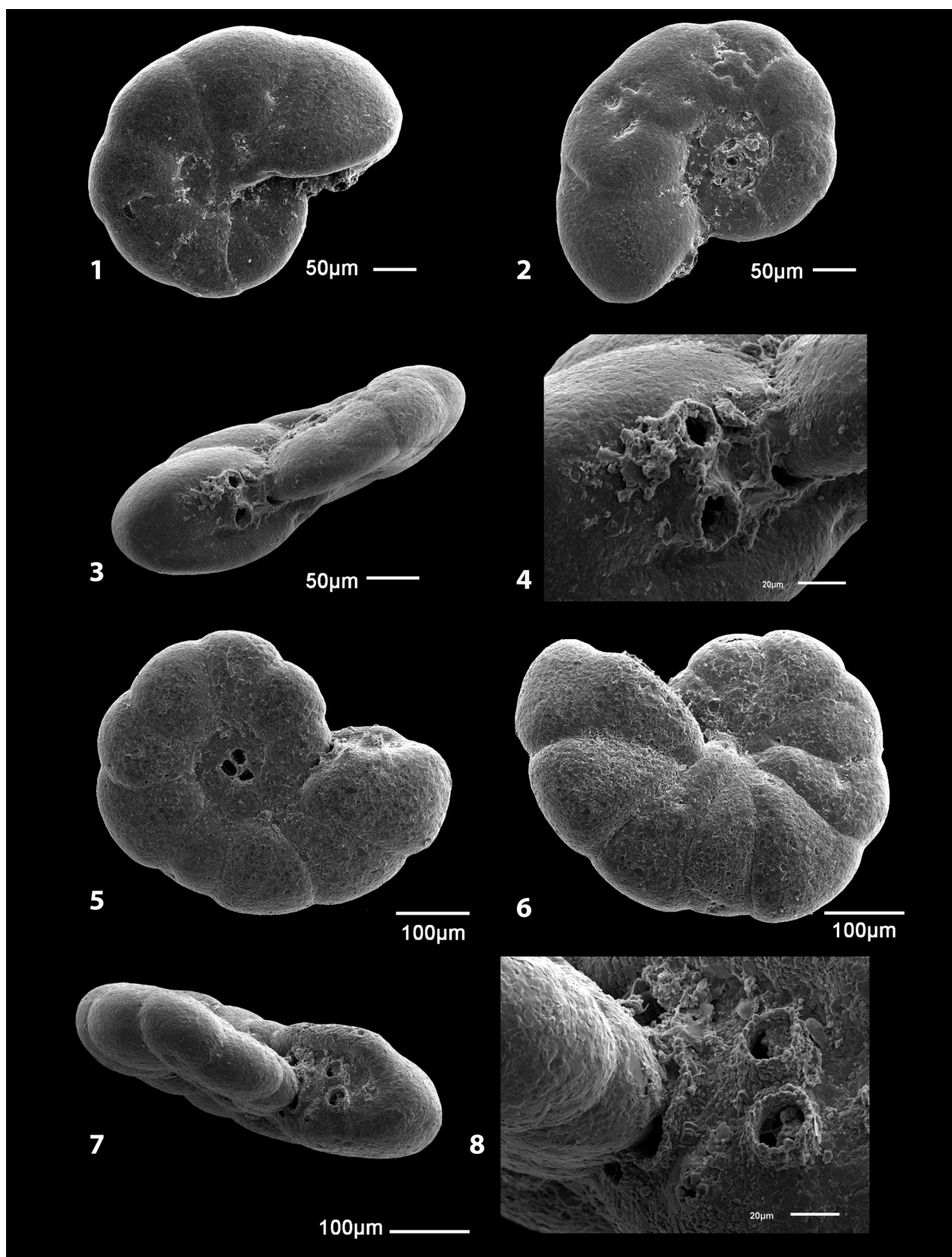


Plate 1: SEM photographs of *Entzia macrescens* and *Entzia* sp. 1-4: *E. macrescens*, Dovey Estuary, GBR. 1. Ventral side 2. Dorsal side 3-4: Apertural face showing primary aperture and supplementary areal openings. 5-8: *Entzia* sp., Camargue, France. 5. Dorsal side 6. Ventral side 7-8: Apertural face showing primary aperture and supplementary areal openings.