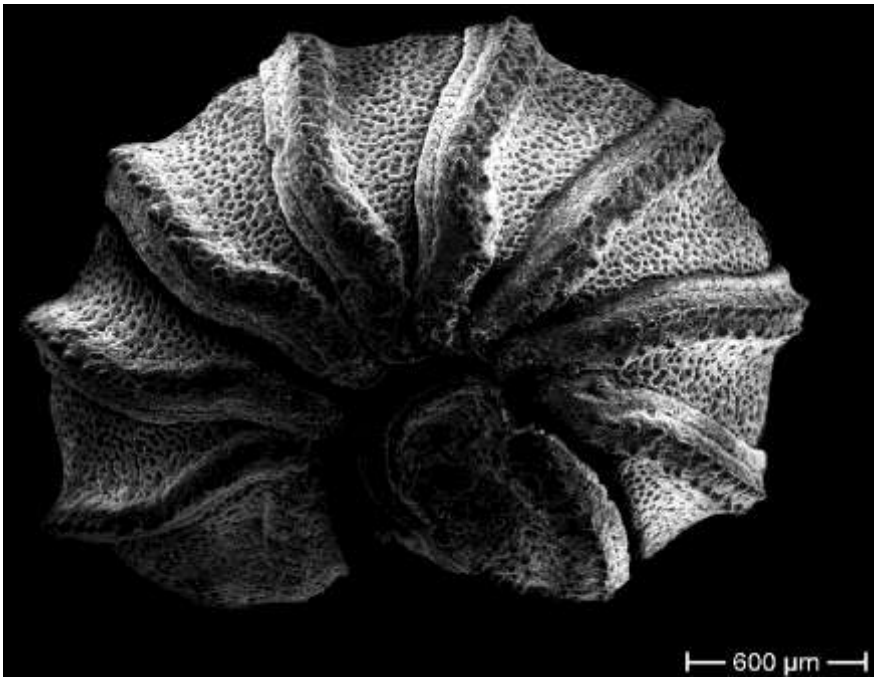
Hymettus

Report on the Origins, Morphological and Genetic Structure of the *Trachysphaera* cf. *lobata* population in the U.K.

2011



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Desmond Kime very helpfully provided specimens of *Trachysphaera* from France, as well as supplying information on *Trachysphaera lobata*, *T. pyrenaica* and *T. drescoi* and their status in France.

Karen Ulmen, Claudia Etzbauer and Jeanne Wilbrandt all helped in the preparation of the scanning electron microscopy images and DNA sequences.

Dr. Axel Schönhofer generously placed his specimens of *Trachysphaera* cf. *drescoi* and *T. pyrenaica* at our disposal. Hans Reip, Jena, generously shared his images of Spanish *Trachysphaera*.

Ian Morgan kindly agreed to collect specimens of *Trachysphaera* from South Wales.

Summary

1. Field work undertaken in February 2011 on the Isle of Wight, England confirmed the continued existence of a population of the pill millipede, *Trachysphaera lobata*. Field work conducted by Ian Morgan in March 2011 confirmed the existence of a *Trachysphaera* population near Llanelli, South Wales. *Trachysphaera lobata* was collected by Desmond Kime from a previously unknown site in the Dordogne, France.

2. Scanning electron microscopy images of various morphological structures taken from 5 specimens from the Isle of Wight population and 8 specimens from the Welsh population, as well as individuals from different French and Spanish *T. pyrenaica* and *T. drescoi* populations revealed a high intraspecific and intrapopulation divergence in some characters, while others were constant; in all 19 specimens were investigated.

3. Multi-layer images taken of 12 specimens from the Isle of Wight and of 9 specimens from the Welsh population revealed large differences in colour and encrustration within each population that could not be correlated to differences in other morphological structures or to genetic differences.

4. DNA was extracted from 13 specimens of *Trachysphaera lobata* from the Isle of Wight population, 15 from the Welsh population, 10 from the French mainland population, and 2 each from two distinct Italian *Trachysphaera* populations. Amplification of the COI gene and sequencing was successful for 2 Italian *Trachysphaera* specimens, all 13 *Trachysphaera* from the Isle of Wight, and 12 *Trachysphaera* from Wales. Due to a contamination, no COI sequences could be obtained from the French *Trachysphaera lobata* population.

5. The genetic data obtained shows no genetic divergence at the population level, but a genetic distance of 1.8% (+/- 0.6%) between the *T. lobata* populations from Wales and the Isle of Wight, is a clear indication of an independent evolutionary history for these two populations. There was no genetic difference in the Isle of Wight population between individuals collected in the main area and at the western end. The genetic distances between the British and the Italian *Trachysphaera* species are very high (25-27%), highlighting the usefulness of the COI mitochondrial gene for separating *Trachysphaera* species and populations, showing that they are separate species.

6. Morphological characters on the last tergite (tergite 10) before the anal shield confirm the identity of both British *Trachysphaera* populations as *Trachysphaera lobata* Ribaut, 1954. However, the SEM study showed few morphological differences between *Trachysphaera pyrenaica* (Ribaut, 1908) and *Trachysphaera lobata* (Ribaut, 1954) aside from the characters on the last tergites. A synonymy of *T. lobata* under *T. pyrenaica* cannot be ruled out.

7. Analysis of the 25 specimens of *Trachysphaera lobata* from Wales found only a single male, but 5 out of 12 dissected female specimens contained eggs indicating ongoing reproduction within the population.

8. Analysis of the 24 specimens of *Trachysphaera lobata* from the Isle of Wight showed a balanced sex ratio, but the three dissected females did not contain visible eggs. One female specimen contained a large parasitic nematode, several times the body length of the *Trachysphaera*.

1. Introduction

Pill millipedes of the genus *Trachysphaera* are very small millipedes with a body length < 4 mm and a heavily sculptured surface, giving them a very unusual appearance (Figures 1A–E). Currently around 30 *Trachysphaera* species are known, most of them with a European distribution from Portugal to the Caucasus (Tabaracu 1988). The northern-most points of their distribution are reached in Britain and southern Germany. Species distribution is patchy, and because of their small size and inconspicuous life style and colour (they resemble tiny calcareous stones) they are rarely encountered by an unskilled or unsuspecting observer.

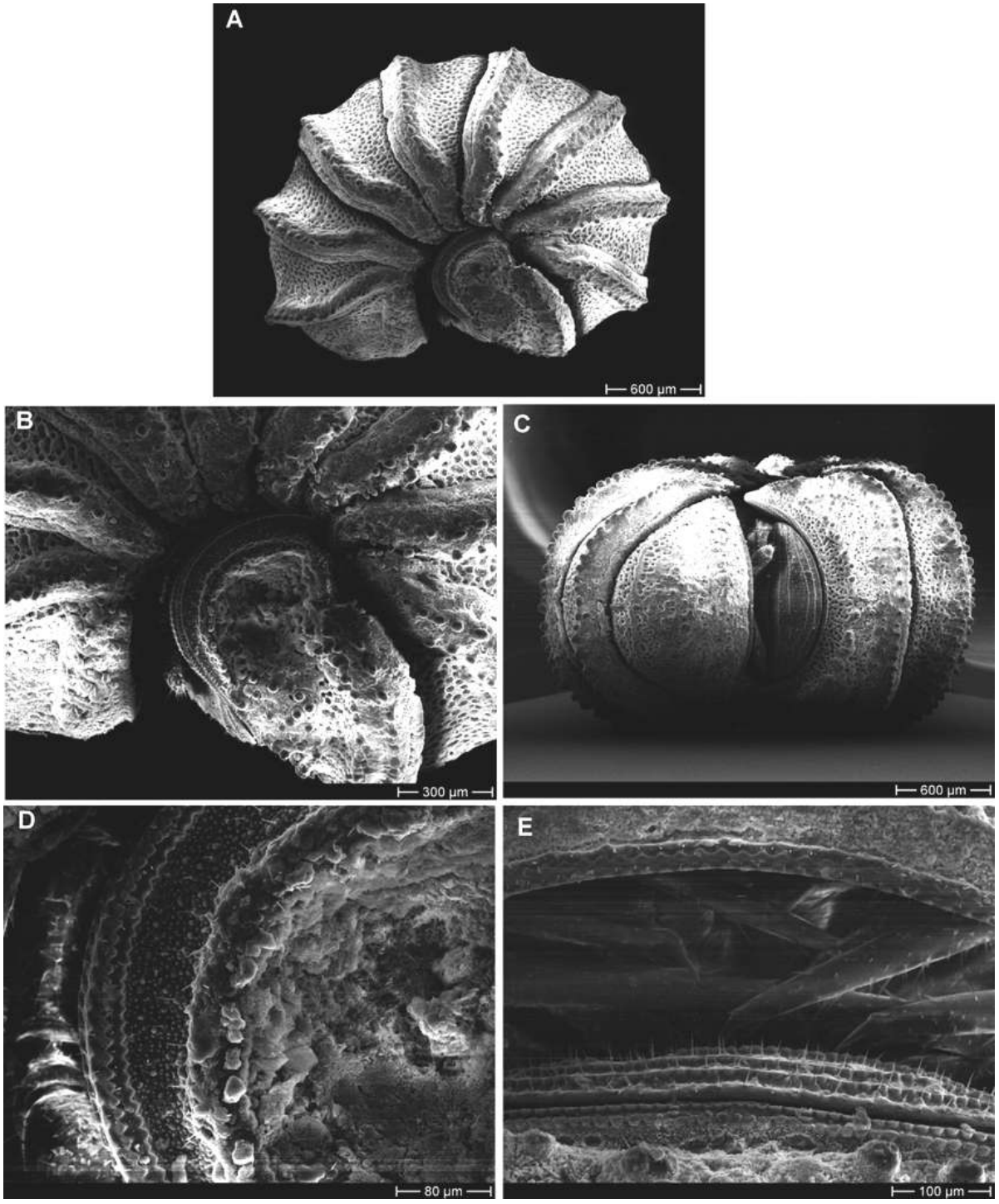

Figure 1: *Trachysphaera* multi-layer photographs and SEM images.

Trachysphaera species are generally difficult to identify, because numerous morphological characters are known to vary greatly both within and between populations, and many species descriptions are based on a very small number of specimens. These variable characters are the reason for the unclear taxonomic status of many *Trachysphaera* species. Historically, the descriptions of the first species of *Trachysphaera* made in the 1930s were based on characters of the external morphology, which were discovered to vary between individuals of the same population and often between both sexes (Attems 1943). After this period species descriptions were based on the male leg pairs 17–19 of which the last are modified to form telopods and paratelopods. In the 1950s (Demange 1959, Conde & Mauriès 1962) it was discovered that these male copulation legs also display a large variation within the same *Trachysphaera* population (Golovatch 1989). Therefore, the taxonomic characters used to separate *Trachysphaera* species from each other are currently ambiguous.

Currently, no DNA sequence data of *Trachysphaera* is available on Genbank; no molecular study has so far been undertaken in this genus.

Trachysphaera was first discovered on the Isle of Wight in 1984 (Jones & Keay, 1986) where it appears to be restricted to a small area of the island near Bembridge. The identity of the English *Trachysphaera* was unclear for a long period, since only female specimens were collected. The English specimens were tentatively determined as *Trachysphaera lobata* (Ribaut, 1954), a species distributed in western France. Recently, a second UK *Trachysphaera* population was discovered in Wales.

During this study we wanted to answer the following questions:

(1) Is the current taxonomic association of the endangered and isolated *Trachysphaera* from the U.K. with *T. lobata* from France justified, or might it be more closely related to populations of *T. pyrenaica* from Spain, or even represent something completely different?

(2) Are there any morphological character differences (structure of the tergites, male copulation legs) within the U.K. populations, or between the U.K. population and the mainland *Trachysphaera*? What is the genetic structure of the endangered *T. lobata* population in the U.K., are the Isle of Wight and Welsh populations different, and how many haplotypes are present?

(3) How is the U.K. *Trachysphaera* population related to the European mainland populations of *T. lobata* and *T. pyrenaica*? Are there any indications that the British *Trachysphaera* populations represent a human introduction from the mainland, or do the British *Trachysphaera* represent one or more relict populations from the Ice Age?

2. Material & Methods

Collecting:
British specimens of *Trachysphaera* were collected by Paul Lee, Steve Gregory and Helen Read on the 22.02.2011 on the Isle of Wight and on 11.03.2011 by Ian Morgan in Wales.

Desmond Kime collected French *T. lobata* from two sites in May 2011: (1) NONTRON, Bois de Bord, mainly Chestnut (*Castanea*) and Oak (*Quercus*) at the collection site, on the side of the gorge of the Rivet Bandiat. Elevation 220 m. Longitude 0°41'09"E, Latitude 45°31'33"N. UTM 10km square E051 N649. (2) CHAMPS ROMAIN, Oak/Chestnut wood in gorge of River Dronne (Natura 2000 site). Elevation 21 0m. Longitude 0°47'06", Latitude 45°31'23". UTM E052 N649. These samples were unfortunately lost on the way from France to Germany. A second batch of mainland *T. lobata* was kindly provided by Mr. Kime, collecting in 08.2011 from mixed deciduous forest in the gorge of the River Auvézère, commune of Génis, Dordogne Department of France. Latitude 45°20'12" North, Longitude 1°08'55" East, Altitude 170 m.

Helen Read also tried to obtain specimens of *T. cf. pyrenaica* from Leitza, Navarra, Spain in 2011, but could not find any specimens. A single specimen collected in 2009 from Leitza and conserved in 70% ethanol was made available by Helen Read.

Additional specimens of *Trachysphaera* were obtained from two sites, (1) *Trachysphaera cf. pyrenaica*, 15 MF, France: Midi-Pyrénées, Dép. Ariège, NW Cazavet, sourroundings of Grotte de l'Estellas, at cliff walls, under stones, logs and sieved from litter, 880 m, N: 42.99665 E: 1.0101, A. Schönhofer leg. 09.10.2009, (2) *Trachysphaera cf. drescoi*, 10 MF, France: Aquitaine, Dép. Pyrénées-Atlantiques, Sare, Grand Grotte de Sare, surrounding decidious forest of *Alnus*, under stones, A. Schönhofer leg. 16.-17.10.2009, 199 m, N: 43.26882 W: 1.57082. These specimens arrived conserved in 70% ethanol

After arrival in the laboratory, all specimens were transferred to individual 2 ml tubes of fresh, 95% ethanol and stored at -20° Celsius.

Multi-layer photographs: Before the destruction of the specimen for the removal of tissue and SEM preparations, multi-layer photographs of the enrolled specimens were taken under a Leica Z6 Imaging-System. A total of 11 specimens from the Isle of Wight population, and 8 from the Welsh population were photographed. For optimal depth of field, the 10-15 single photographs taken from each specimen were put together into one multi-layer photograph using the software Auto-Montage.

Scannning electron microscopy: A total of 19 specimens of *Trachysphaera* were studied using scanning electron microscopy. These 19 specimens included five specimens of *T. lobata* from the Isle of Wight, eight of *Trachysphaera* sp. from Wales, four of *T. pyrenaica* (one from Spain, three from France), and two of *T.* cf. *drescoi* (from the French Pyrenees close to the type locality).
Objects prepared for SEM were: (1) male telopods; (2) anal shield; (3) anterior body part including head, collum, thoracic shield and tergite 3, (4) midbody tergite. For scanning electron microscopy, samples were cleaned manually and dehydrated in an ethanol series (80%, 90%, 95% and twice in 100%) and air-dried overnight. The samples were then mounted on aluminium stubs before being coated with gold in a sputter coater for 240 seconds. SEM micrographs were taken using a Hitachi S2460N SEM, based at the ZMFK. Ultrasonic cleaning of the often dirty samples was not attempted, since the risk of destroying

the unique surface structure of the tergites was deemed too high. The same specimens used for the SEM study were also used for DNA extraction so that the observed morphological differences could be directly compared with the genetic differences.

DNA extraction: DNA was extracted from a total of 42 specimens (see Table 1). For DNA extraction, muscle tissue was removed from the tergites of the specimens, often during the preparation of the SEM objects. Because of the tiny size of the specimens (most were 3 mm long and 1.5 mm wide) it was very difficult to obtain enough muscle tissue. Because of the low DNA yield of previous extractions of *Trachysphaera*, the inclusion of any calcareous tergite structure into the extraction samples was avoided as far as was possible. DNA extraction was undertaken using the Qiagen spin column DNeasy Blood&Tissue kit using the standard protocols.

Table 1: DNA extractions and voucher number

#	Morphological voucher	Details
TW1	Helen 3	Trachysphaera F Isle of Wight W-End
TW2	Helen 4	Trachysphaera F Isle of Wight main area
TW3	Helen 5	Trachysphaera Isle of Wight main area
TW4	Helen 27	Trachysphaera Wales
TW5	Helen 28	Trachysphaera Wales
TW6	TWMAD 188a	Trachysphaera F Oropa
TW7	TWMAD 188b	Trachysphaera F Oropa
TW11	Helen 2	Trachysphaera Isle of Wight W-End
TW12	Helen 6	Trachysphaera Isle of Wight main area
TW13	Helen 7	Trachysphaera Isle of Wight main area
TW14	Helen 8	Trachysphaera Isle of Wight main area
TW15	Helen 9	Trachysphaera Isle of Wight main area
TW16	Helen 10	Trachysphaera Isle of Wight main area
TW17	Helen 11	Trachysphaera Isle of Wight main area
TW18	Helen 12	Trachysphaera Isle of Wight main area
TW19	Helen 13	Trachysphaera Isle of Wight main area
TW20	Helen 14	Trachysphaera Isle of Wight main area
TW21	Helen 30	Trachysphaera Wales
TW22	Helen 31	Trachysphaera Wales
TW23	Helen 32	Trachysphaera Wales
TW24	Helen 33	Trachysphaera Wales
TW25	Helen 34	Trachysphaera Wales
TW26	Helen 35	Trachysphaera Wales
TW27	Helen 36	Trachysphaera Wales
TW28	Helen 37	Trachysphaera Wales
TW29	Helen 38	Trachysphaera Wales
TW30	Helen 39	Trachysphaera Wales
TW31	Helen 40	Trachysphaera Wales
TW32	Helen 41	Trachysphaera Wales
TW33	Helen 42	Trachysphaera Wales
TW47	TW47	Dordogne, T. cf. lobata, hell F
TW48	TW48	Dordogne, T. cf. lobata, dunkel F
TW49	TW49	Dordogne, T. cf. lobata, hell F
TW50	TW50	Dordogne, T. cf. lobata, hell F (bad)
TW51	TW51	Dordogne, T. cf. lobata, dunkel F w/eggs
TW52	TW52	Dordogne, T. cf. lobata, dunkel F w/eggs
TW53	TW53	Dordogne, T. cf. lobata, hell, F, klein
TW54	TW54	Dordogne, T. cf. lobata, dunkel F
TW55	TW55	Dordogne, T. cf. lobata, hell F
TW56	TW Mad 133	Trachysphaera Storo F
TW57	TW57	Dordogne, T. cf. lobata, hell F
TW58	TW Mad 133	Trachysphaera Storo M

All specimens from which DNA was extracted were given an individual voucher number and were transferred to the collections of the ZFMK.

Molecular analysis:
Polymerase chain reactions (PCR) was used to amplify a fragment of the mitochondrial COI gene. Qiagen PCR Multiplex mix was used to amplify the COI gene with the standard HCO/LCO primers. All reactions were cycled once for 15 min at 95°C and then with a touch-down PCR 15 (25) cycles of 35 s at 94°C, 90 s at 55 (40) °C, and 90 s at 72°C. PCR bands were generally weak for *Trachysphaera* specimens, probably because of the minute amount of tissue used for extraction (see above). Successful PCR reactions were purified following the manufacturers protocol for the QIAquick PCR Purification Kit. Purified PCR products were outsourced for double-string sequencing to a contract sequencing facility (Macrogen, Seoul, Korea) on an ABI3730 XL automatic DNA sequencer, using the same primer sets as for PCR. Sequencing reads were assembled with the program Seqman™ II (DNASTAR, Inc.) while the identity of all new sequences was confirmed with BLAST searches (Altschul et al. 1990).

COI sequences could be obtained of a total 31 specimens, but after using Blast to verify the samples, four were discovered to be contaminations. The total number of successfully sequenced specimens was 2 *Trachysphaera* sp. (outgroup) from Italy, 13 *Trachysphaera lobata* from the Isle of Wight, England, and 12 *Trachysphaera lobata* from Wales.
Sequences were aligned by hand in Bioedit, and the number of indels, transitions and transversions were counted by eye. Mean pairwise distances between haplotypes and between populations were determined using MEGA (v. 5, Tamura et al. 2011).
The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei 1993). The tree with the highest log likelihood (-1912.9746) is shown (Fig. 8). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 668 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

3. Results

Morphology
The multi-layer photographs of the *Trachysphaera* specimens from the Isle of Wight (Fig. 2 A–D) and from Wales (Fig. 2 E–H) show large differences in colour and encrustation with soil in each population.

Scanning electron microscopy results were inconclusive, a further analysis of the numerous pictures (around 400!) is still necessary. For the moment, the focus will be placed on the anal shield on the head and collum (Fig. 3), the endotergum (underside of the posterior margin of the tergites, Fig. 4), and the anal shield and last tergite in lateral view (Fig. 5), posterior view (Fig. 6), and the posterior margin of the anal shield (Fig. 7).

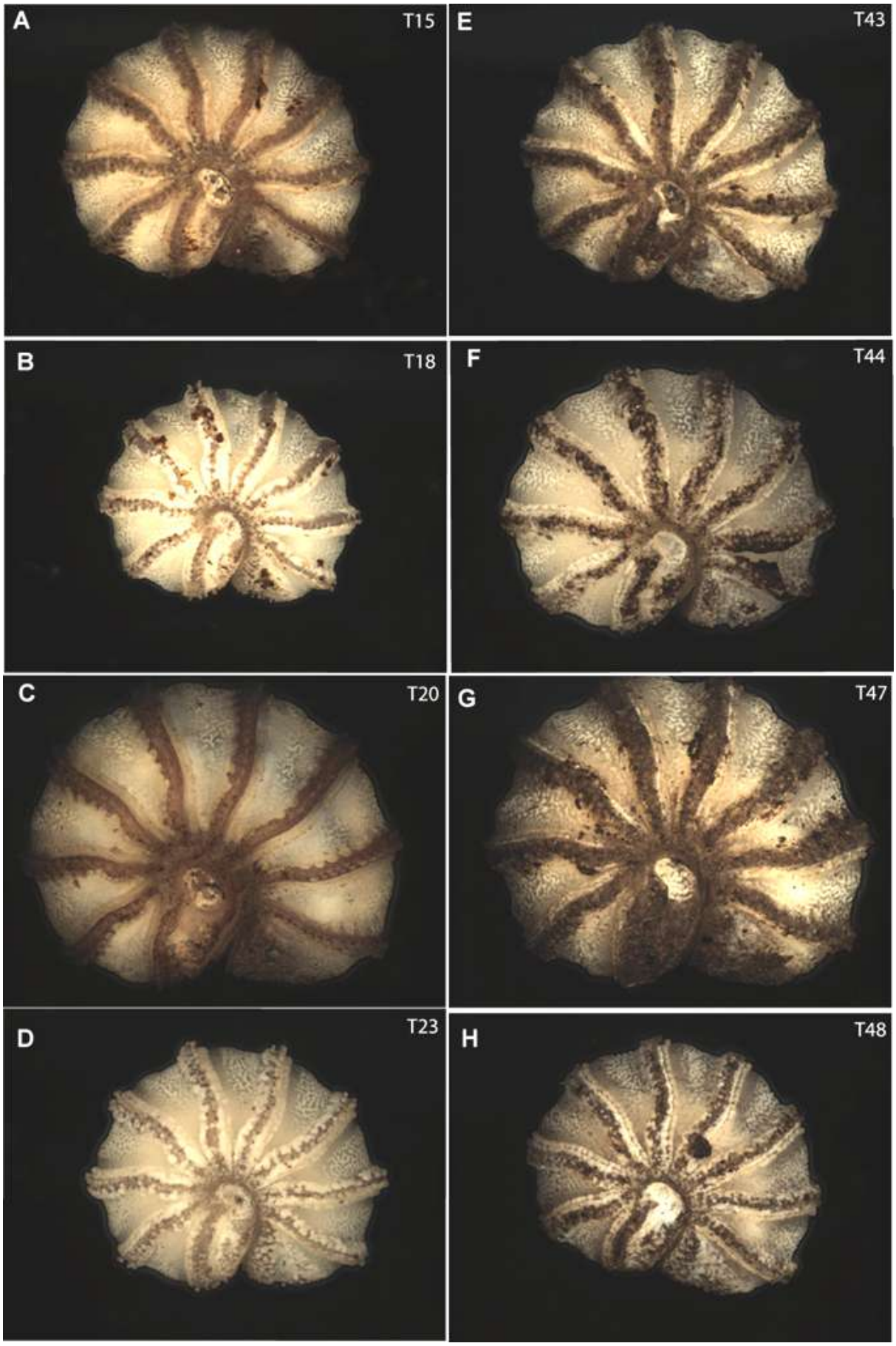

Figure 2: *Trachysphaera lobata* (Ribaut, 1954) multi-layer photographs, **A–D** population from the Isle of Wight, England; **E–H** population from Wales. Not to scale.

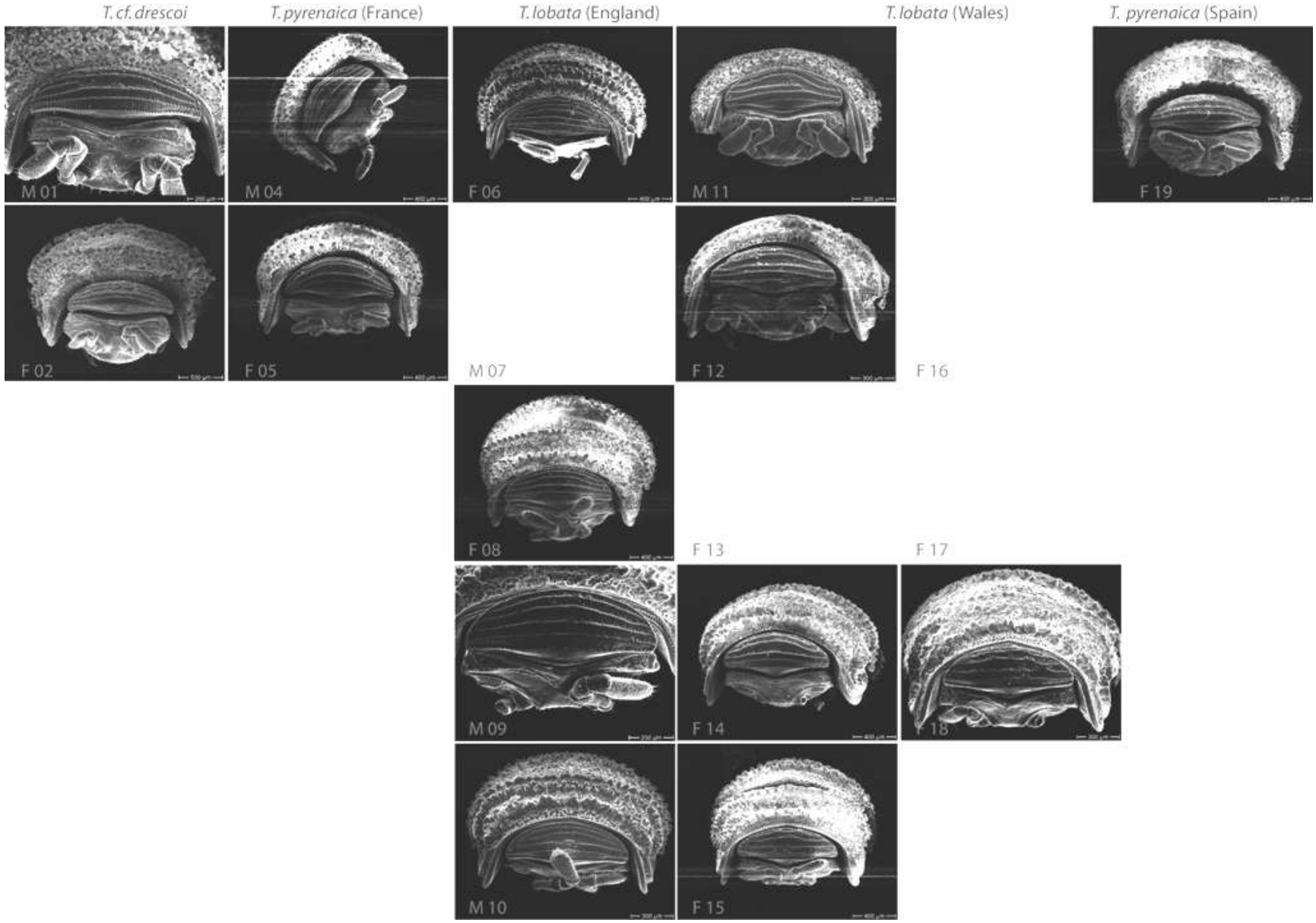

Figure 3: SEM images of head, collum and thoracic shield, frontal view. M = male, F = female.

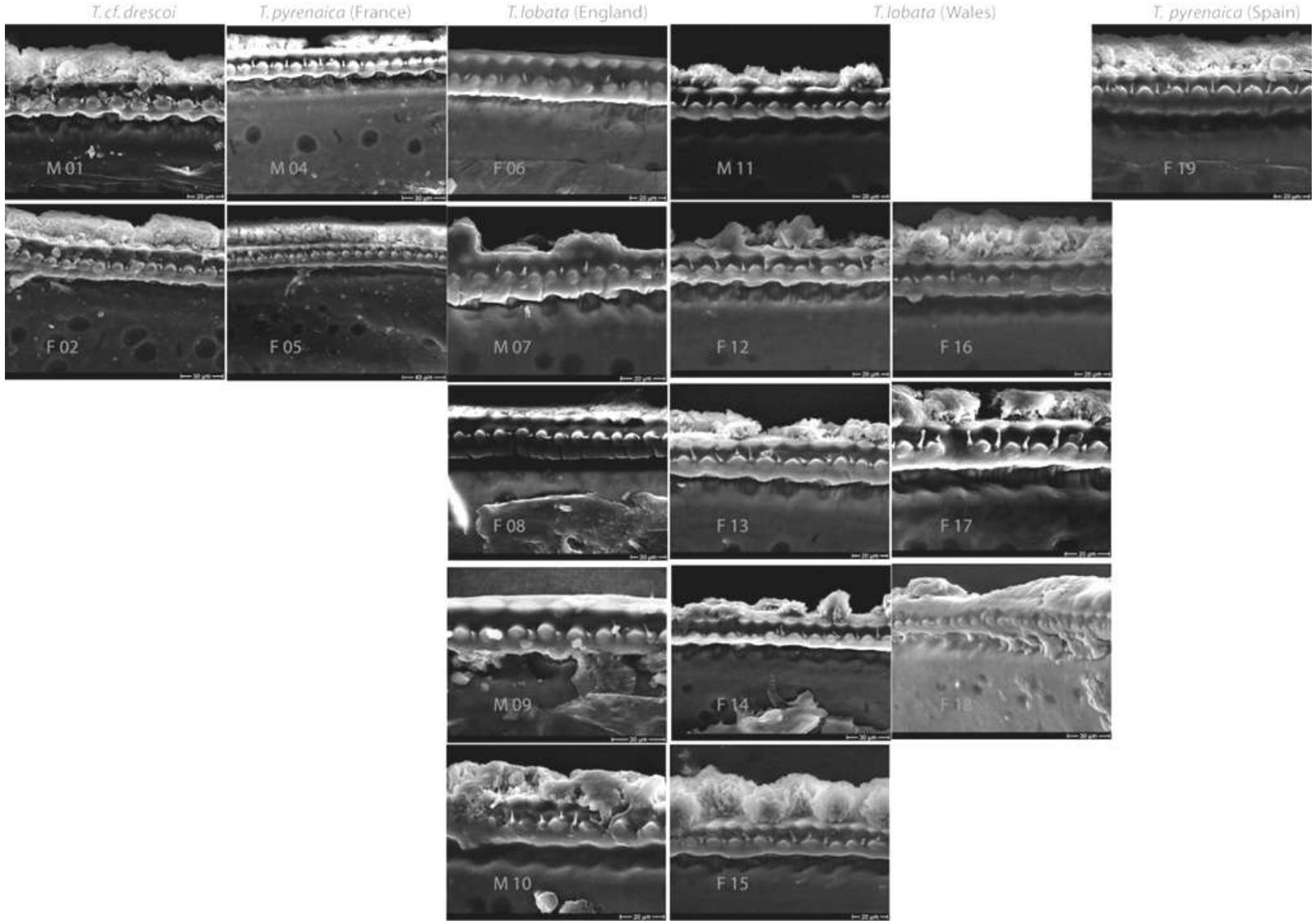

Figure 4: SEM images of the endotergum (underside of the posterior margin of the tergites). M = male, F = female.

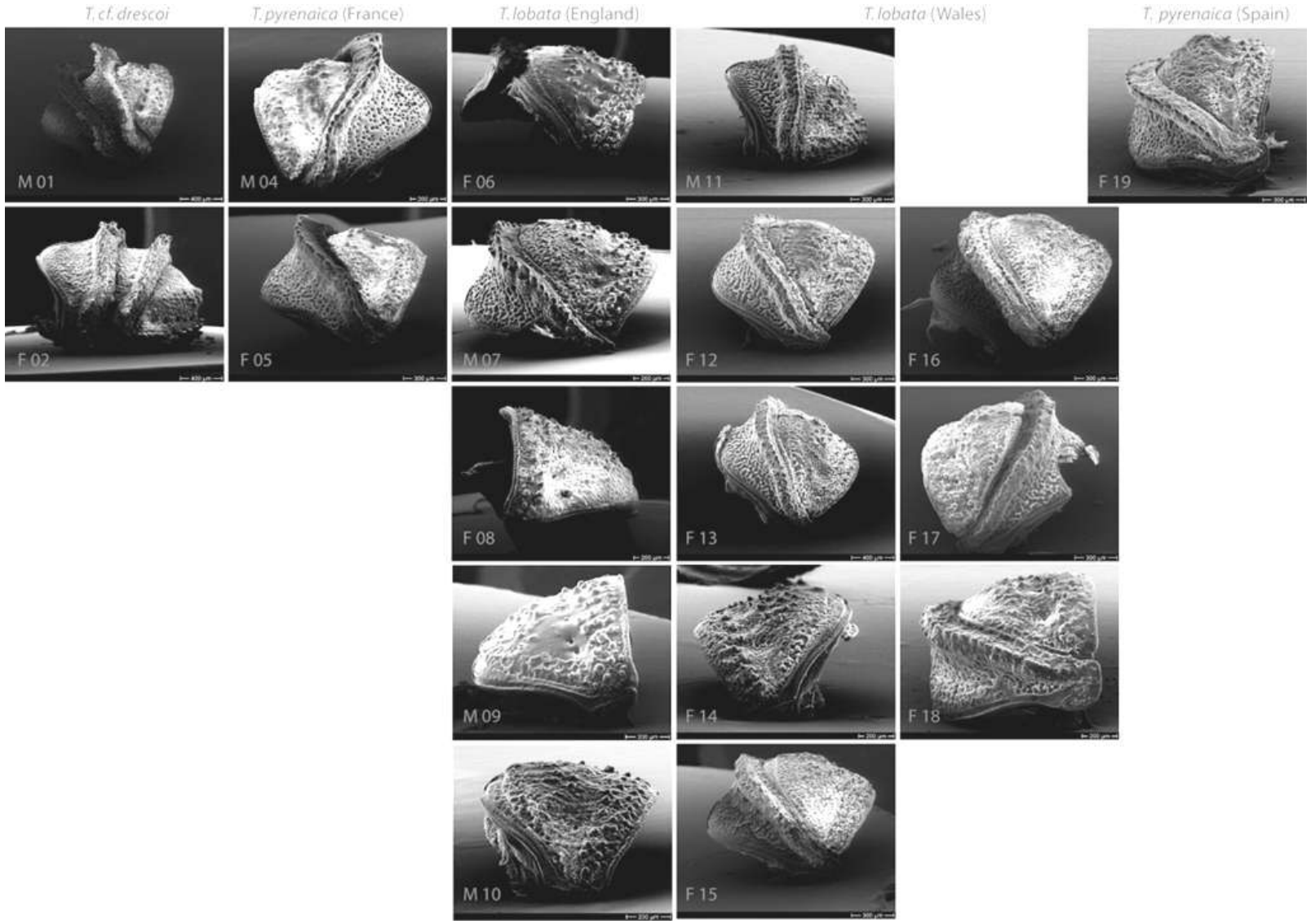

Figure 5: SEM images of the anal shield, often with T10 still attached, lateral view. M = male, F = female. *Trachysphaera lobata* differs from *T. pyrenaica* and *T. drescoi* in the presence of two rows of nodules on the crest of tergite 10 (one in the other species).

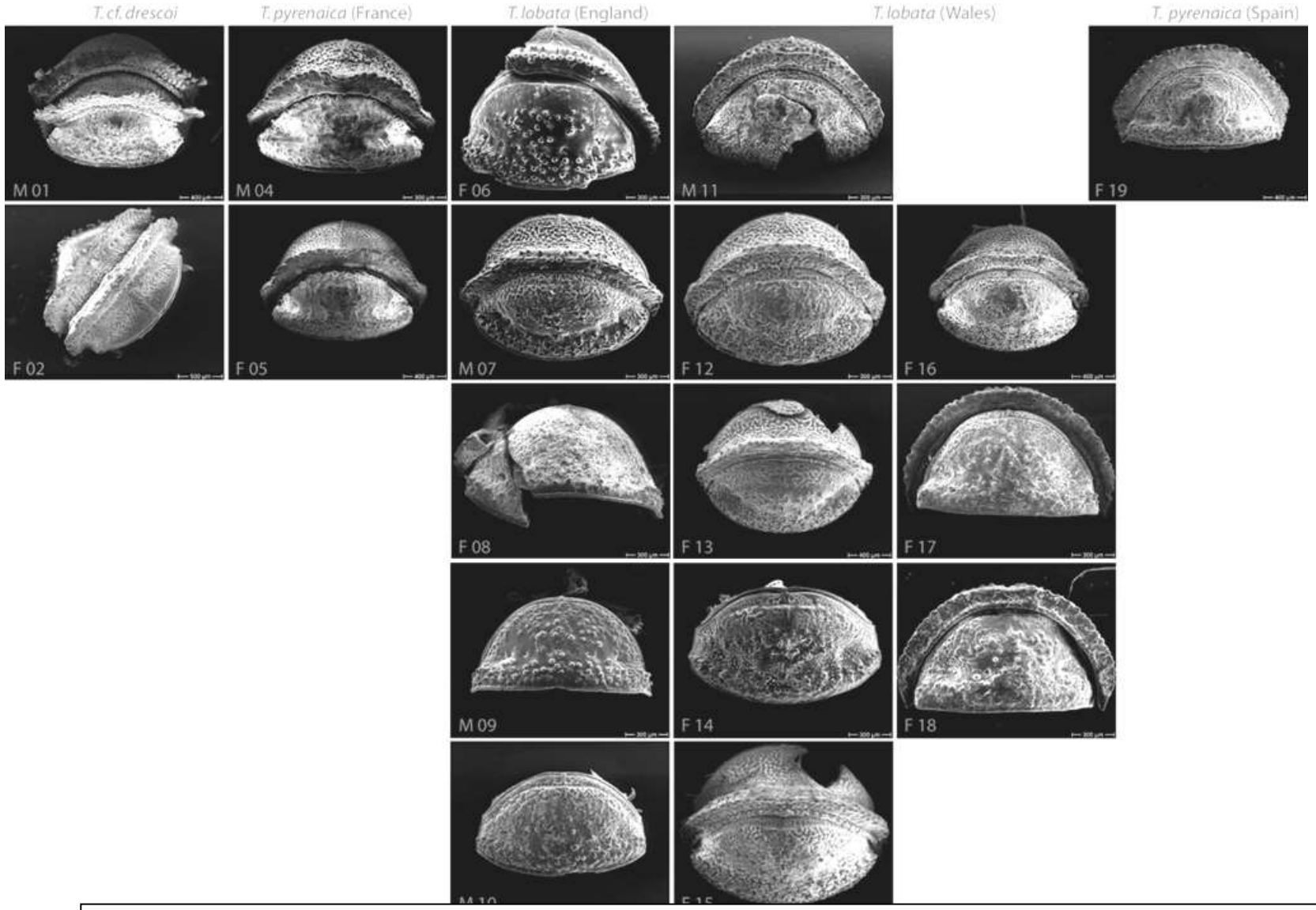

Figure 6: SEM images of the anal shield, often with T10 still attached, posterior view. M = male, F = female.

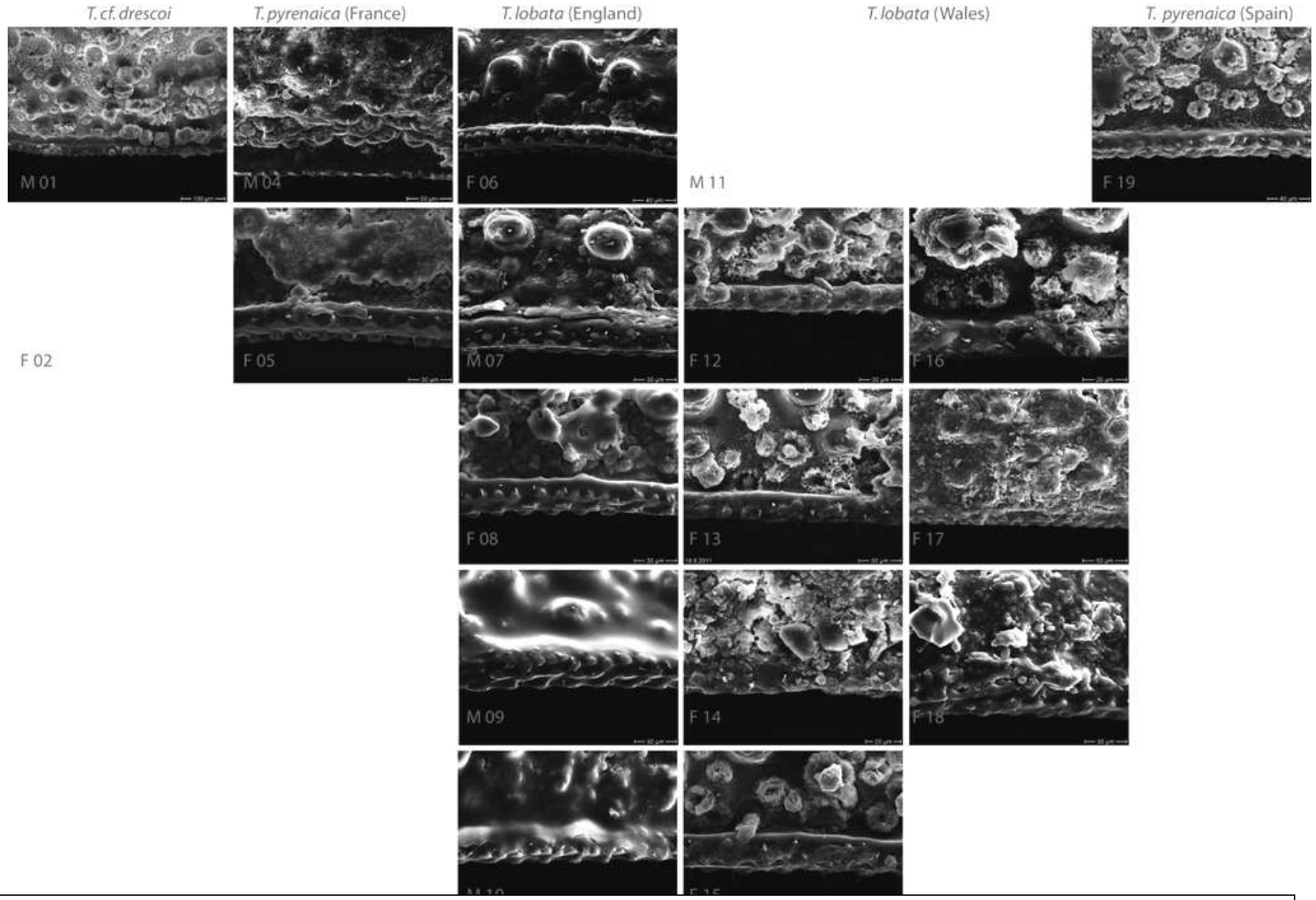

Figure 7: SEM images of the anal shield, posterior view, detail of marginal structures. M = male, F = female.

Molecular analysis:

No variation of the COI sequence was found between individuals from the same *Trachysphaera* population. However, considerable differences were found between the population of *T. lobata* from the Isle of Wight (England) and the Welsh population. Both populations differ at 1.8% (+/- 0.6) of their COI sequence (Table 2). The differences between *Trachysphaera lobata* and the *Trachysphaera* species from Italy, are much higher (17.4–17.8%). Even larger are the differences between the pill millipede genus *Trachysphaera* and those of *Polyzonium* of the order Polyzoniida (>80%).

Table 2: **Estimates of Evolutionary Divergence between COI Sequences.** The number of base substitutions per site are shown. Standard error estimate(s) were obtained by a bootstrap procedure (500 replicates). Analyses were conducted using the Maximum Composite Likelihood model. The analysis involved 29 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 667 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

Species 1	**Species 2**	**Distance [%]**	**Std. Err**
Polyzonium	Trachysphaera Italy	83.2	226.7
Polyzonium	T IsleofWight	85.8	240.5
Trachysphaera Italy	T IsleofWight	17.8	6.4
Polyzonium	T Wales	84.9	146.4
Trachysphaera Italy	T Wales	17.4	5.8
T IsleofWight	T Wales	1.8	0.6

Between the *Trachysphaera* populations from the Isle of Wight and Wales, a total of 12 base pair changes occurred, including one transition and 11 transversions (Table 3). These changes were all silent mutations and did not result in any amino acid changes.

Table 3: **Changes in the base pairs of the COI sequence between the Isle of Wight *T. lobata* and the Welsh population.** The number of base substitutions per site from between sequences are shown.

Base #	***T. lobata* (Isle of Wight)**	***T. lobata* (Wales)**	**Transition (TS) / Transversion (TV)**
39	C	A	TV
116	A	G	TS
243	T	C	TS
255	C	T	TS
294	A	G	TS
327	T	C	TS
330	T	C	TS
336	T	C	TS
429	T	C	TS
438	C	T	TS
460	G	A	TS
465	G	A	TS

The phylogenetic maximum likelihood tree (Fig. 8) clearly shows the sister-clade relationship between the *T. lobata* populations from the Isle of Wight and Wales. As indicated by the Distance Matrix (Table 2), *T. lobata* is quite distantly related to the Italian *Trachysphaera* (Fig. 8).

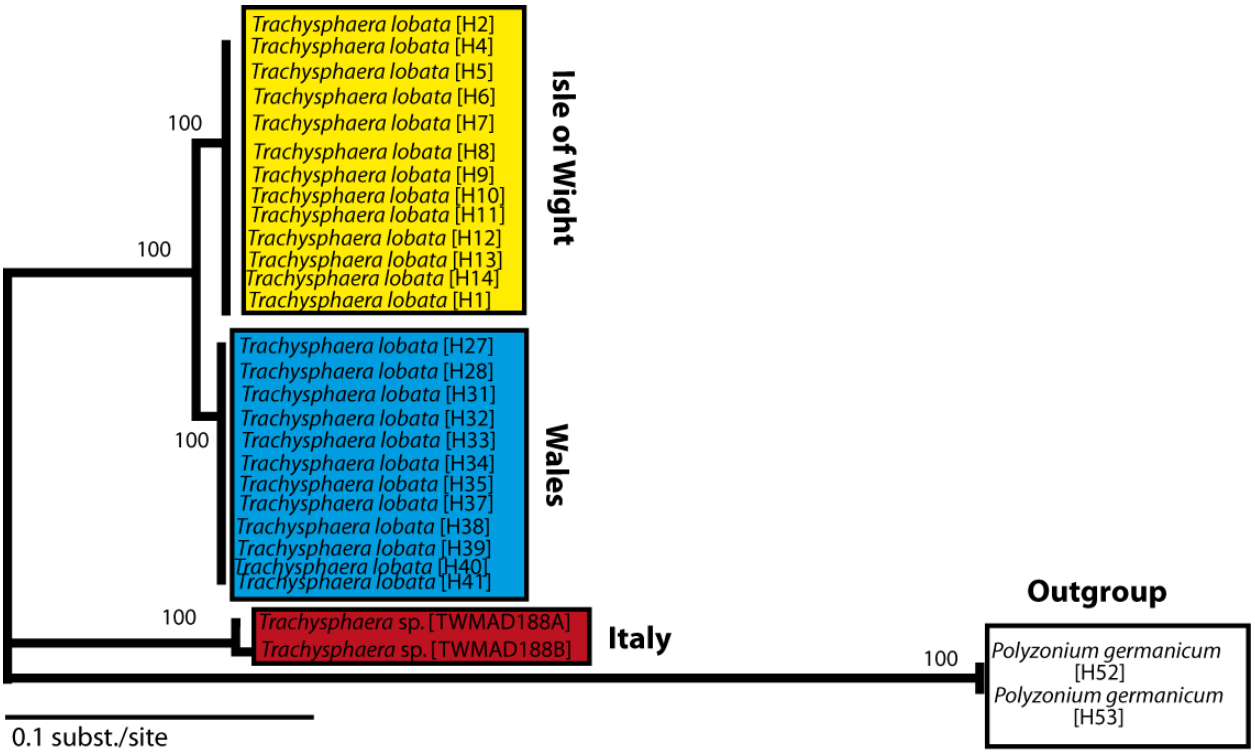

Figure 8: Maximum likelihood Tree computed by MEGA5 after the Tamura-Nei model. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage (>50%) of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 667 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

Conclusions

It can be concluded that the two British populations of *Trachysphaera lobata*, while related to each other, consist of separate units, with a different evolutionary history. The question of whether (1) the two disjunct populations are the last remaining relict populations of a *Trachysphaera* distribution spanning southern England and South Wales, with all intermediate population (and haplotypes) either undiscovered or extinct, or (2) if the Welsh and Isle of Wight populations are the result of two separate dispersal or introduction events from distant European mainland populations, cannot be answered because of the lack of samples from the European continent. Careful sampling of European populations would clarify whether the haplotypes of the Welsh and Isle of Wight *Trachysphaera* can be found on the European mainland.

This study presents a new foundation for (1) numerous morphological characters collected from SEM images, as well as (2) the first genetic data of a *Trachysphaera* species. In the future, data from other

populations and species of *Trachysphaera* can be added to complete the picture we currently have of this interesting genus of tiny pill millipedes so perfectly adapted to life inside the soil.

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