Journal of Foraminiferal Research, v. 53, no. 2, p. 177–181, April 2023

QUANTITATIVE SURFACE MORPHOLOGY OF *AMMONIA* CF. *BECCARII* AND *AMMONIA PARKINSONIANA* BY ATOMIC FORCE MICROSCOPY: TAXONOMIC POTENTIAL

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ABSTRACT

Atomic Force Microscopy (AFM) is used to quantify the surface morphology of two benthic foraminiferal species. Scanning Electron Microscopy (SEM) is traditionally used for surface ultrastructural studies but cannot estimate three-dimensional measurements. Therefore, AFM is used to measure pore depth and surface roughness, as well as two-dimensional features such as pore density, pore diameter, and porosity. Specimens of *Ammonia* cf. *beccarii* and *Ammonia parkinsoniana* have been analyzed in this study. The species have been compared in this pilot study by mapping the test structure in three dimensions. The two species have a distinct topographical contrast and significant differences in pore density, diameter, porosity, depth, and surface roughness. It is demonstrated here that AFM can be used to understand the morphological differences with finer details between the species of foraminifera. The effect of the environment on the estimated surface parameters should be investigated.

INTRODUCTION

Ammonia Brünnich (1772), a benthic foraminiferal genus with a cosmopolitan distribution, inhabits shallow marine, lagoonal, and estuarine environments in the warm-temperate and tropical zones (Walton & Sloan, 1990; Sen Gupta, 2007). *Ammonia* along with its morphotypes is one of the dominant taxa found in Chilika Lagoon on the east coast of India (Dasgupta & Ghosh, 2021), the largest brackish water lagoon on the Asian continent and the first recognized Ramsar site in India. There is a dilemma in the identification of the correct *Ammonia* species due to its large morphological variation (Hayward et al., 2004). Different species of *Ammonia* are characterized by contrasting pore geometry and characters (Petersen et al., 2016). Pore variability is also quite noticeable at the interspecies level (Gooday & Alve, 2001). Dasgupta & Ghosh (2021) distinguished different species of *Ammonia* based on the pore geometry as a distinct morpho-character. In a foraminiferal study, molecular and morphological analysis have been used extensively to study the taxonomy of *Ammonia* and other genera for species identification (Darling et al., 2016; Bird et al., 2020). One such study was conducted by Hayward et al. (2021) who studied the global geographical distribution of living *Ammonia* based on molecular and morphological approaches. Identification of the correct foraminiferal species is significant for the accuracy of analytical results. So, it is crucial in any foraminiferal study to correctly identify the species.

Frequently, molecular and morphometric analyses are used in combination for close species identification, such as for the genus *Ammonia*. But for fossil specimens where genetic analysis could be restricted or is unfeasible (Richirt et al., 2019a), a morphometric approach for species characterization is utilized. Based only on two morphological characteristics, the sutural height on the spiral side and the pore diameter on the penultimate chamber, the phylotypes of *Ammonia* can be distinguished accurately (Richirt et al., 2019a). The application of biometric analysis can be found in the study conducted by Schönfeld et al. (2021), used for the identification of *Ammonia* species, without dependence on genetic analysis. The reliability of morphometric analysis can develop further with improved analytical techniques.

The use of an Atomic Force Microscope (AFM) has been recently incorporated as a tool for foraminiferal research. The study of elemental banding on the surface of *Amphistegina lessonii*, *Ammonia tepida* (Geerken et al., 2019), and the quantitative surface morphology of *Ammonia tepida* (Giordano et al., 2019) are the only foraminiferal applications of AFM to date.

This work shows the new morphological comparison technique with a three-dimensional approach, focused on the pore characters of the species *Ammonia* cf. *beccarii* (Linnaeus, 1758) and *Ammonia parkinsoniana* (d'Orbigny, 1839), which has not been carried out before. We measured the following morphological parameters: Pore density (PD) is the ratio of pore count per measurement frame (μm^{-2}); Pore diameter; Porosity (Po) is the percentage of the pore surface in the measurement frame; Pore depth (Pde) is the minimum height of the pore measured in terms of the average height; Surface roughness (R) is the measurement of deviation or irregularities of surface texture.

In our study, the use of AFM analysis has yielded quite promising results on the morphological aspect of benthic foraminifera. It has the potential to advance the morphometric analysis of *Ammonia* in much more detail than through using only SEM images. Consequently, accuracy in species identification improves with an increase in better morphometric-quantification techniques.

MATERIALS AND METHODS

A 52-cm long core was taken from the central sector of Chilika Lagoon (19°41'58.78"N, 85°23'19.53"E). The protocol of Schönfeld et al. (2012) was followed for the benthic foraminiferal study. Our work is based on the core-top sample, at 0–1 cm core depth. Specimens of *Ammonia* cf. *beccarii* and *Ammonia parkinsoniana* were picked using a stereo-zoom microscope (Nikon SMZ1000). The analyzed species are shown in Figure 1. The taxonomic status of

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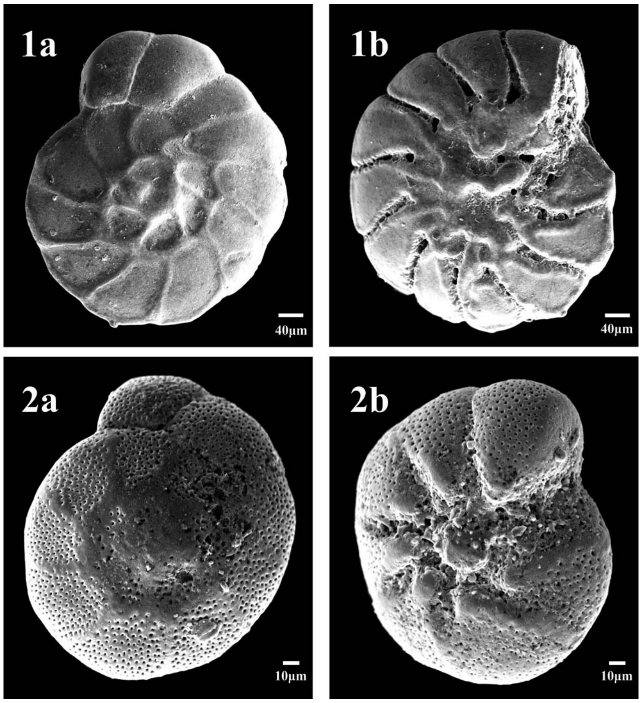

FIGURE 1. Microphotographs of the species from an electron microscope which have been analyzed under AFM (Atomic Force Microscope). **1**a *Ammonia* cf. *beccarii* (Linnaeus), spiral view, **1b** umbilical view. **2**a *Ammonia parkinsoniana* (d'Orbigny), spiral view, **2b** umbilical view.

a foraminiferal genus was identified based on Loeblich & Tappan (1987), and the species were determined by their morphological attributes, described by Dasgupta & Ghosh (2021) and Hayward et al. (2021). *Ammonia beccarii* and *Ammonia parkinsoniana* have been previously reported from the Chilika Lagoon on the east coast of India. Rao et al. (2000) have described the foraminiferal species from the Chilika Lagoon consisting of the two species mentioned here.

Each specimen was carefully mounted on a microscopic cover glass (18 mm) and fixed by double adhesive tape. As the spiral or dorsal side displays the maximum number of pores, it was faced upwards. The final or ultimate chamber (n) on some specimens, due to the physical alteration, was found to be relatively broken. Hence, the penultimate chamber (n–1) was analyzed.

The analysis was conducted in the AFM facility at S.N. Bose National Centre for Basic Sciences, Kolkata. Topographical three-dimensional mapping was done using the AFM model di INNOVA, Bruker. A cantilever (rectangular), antimony (n) doped Si, model RTESPA-300, Bruker ($T = 3.4$ µm; $L = 125$ µm; $W = 40$ µm; $f_o = 300$ kHz) was operated on a tapping mode (Sample/line = 256; Scan rate = 0.5000 Hz; Scan Range = 15 µm). The penultimate chamber (n–1) is considered for the analysis as representative of the test. The size of the AFM scan of 15×15 µm^2 was chosen for the measurement. Along with the characteristic topographical structure, pore characteristics such as pore density (PD), pore diameter (Pdi), porosity% (Po), pore depth (Pde), and surface roughness (R) can be calculated in detail as described by (Giordano et al., 2019). Petersen et al. (2016) de-

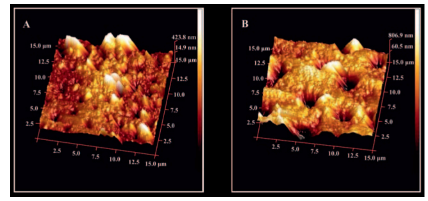

FIGURE 2. The topographical map is shown as a 3D AFM image. **A** *Ammonia* cf. *beccarii.* **B** *Ammonia parkinsoniana* (scan area = 225 µm^2).

vised a standard measurement method for pore patterns that has been followed for the study.

The AFM data were acquired using NanoDrive v8 real-time control and NanoScope Analysis software. Data processing was done with PAST (Paleontological Statistics; Hammer et al., 2001) and ImageJ (Schneider et al., 2012) software.

RESULTS AND DISCUSSION

A total of eight specimens, consisting of four *Ammonia* cf. *beccarii* and four *Ammonia parkinsoniana* were analyzed under the AFM. A total of 136 pores were analyzed (*Ammonia* cf. *beccarii* = 100; *Ammonia parkinsoniana* = 36) for the various pore parameters, out of which 24 pores were specifically used for depth analysis.

A three-dimensional topographic map for the species *Ammonia* cf. *beccarii* and *Ammonia parkinsoniana* is shown in Figure 2. A distinct contrast in species-specific pore patterns is evident. *Ammonia* cf. *beccarii* shows a reduced value of Pdi (0.564 µm) and an enhanced PD (0.155/µm^2). *Ammonia parkinsoniana* shows a significant increase in Pdi (1.629 µm) and a drastic reduction in PD (0.035/µm^2) values (Pdi and PD are based on average calculated values). Calculated pore parameters are shown in Table 1.

Pore depths (Pde) for the representative area (225 µm^2) were measured at a linear distance (microns) for the species *Ammonia* cf. *beccarii* and *Ammonia parkinsoniana* as shown in Figure 3. *Ammonia* cf. *beccarii* shows an average value of Pde (–0.041 µm); *Ammonia parkinsoniana* has a Pde (–0.116 µm) on average. The negative sign in the Pde value implies the pore height measured below the average topographical height. The value of Pde in *Ammonia parkinsoniana* is significantly higher, ~2–3 times that in *Ammonia* cf. *beccarii*. Although the Pdi is different from those in previous studies, it will be possible to accurately determine the species by comparing the PD and surface roughness (R) revealed by the AFM-based method in this study along with genetic analysis. A comparative profile of Pdi and Pde for the studied species is shown in Figure 4.

Another parameter possible only through AFM is surface roughness (R), which was also measured. *Ammonia* cf. *beccarii* shows an average surface roughness value of Ra = 0.092 µm, whereas *Ammonia parkinsoniana* has a value of Ra = 0.206 µm. Consequently, it supports the idea that pore density (PD) has an inverse relation to surface roughness (R) as evidenced by the measured data.

The corresponding data set of pore parameters from Table 1 has been normalized to a percentage value out of 100. The data conversion of the pore parameter is based on

TABLE 1. Measurement of pore parameters of *Ammonia* cf. *beccarii* and *Ammonia parkinsoniana.* The data on *Ammonia tepida* by Giordano et al. (2019, tables 1–3) has been averaged for comparison study.

No.	Parameters	*Ammonia* cf. *beccarii*	*Ammonia parkinsoniana*	*Ammonia tepida*
1	Pore density (PD)	0.155/µm^2	0.035/µm^2	0.155/µm^2
2	Pore diameter (Pdi) average	0.564 µm	1.629 µm	1.024 µm
3	Porosity% (Po)	3.150%	8.775%	4.791%
4	Pore depth (Pde)	–0.041 µm	–0.116 µm	–0.42 µm
5	Roughness average (Ra)	0.092 µm	0.206 µm	0.067 µm

a simple computation:

$$(a + b + c) = t$$

where t is the sum total of all the species (a = species 1, b = species 2, and c = species 3). Pde is taken as positive, and the final calculated value is given by:

$$\text{species } 1 = (at^{-1}) \times 100$$
$$\text{species } 2 = (bt^{-1}) \times 100$$
$$\text{species } 3 = (ct^{-1}) \times 100$$

For comparison, AFM data of *Ammonia tepida* has been incorporated in our work from Giordano et al. (2019, pl. 5–6, tables 1–3) and has been averaged. The comparative chart is shown in Figure 5. The morpho-parameters pore density (PD), pore diameter (Pdi), porosity% (Po), pore depth (Pde,) and surface roughness (R) are compared among the species *Ammonia* cf. *beccarii*, *Ammonia parkinsoniana* and *Ammonia tepida*. From Figure 4, *Ammonia parkinsoniana* has a relatively lower pore count, so the pore density (PD) has a reduced value compared to the other two species. But the lower (PD) value is compensated for by the increase in pore diameter (Pdi). Such interrelated pore characteristics are also noticed for the other two species *Ammonia* cf. *beccarii* and *Ammonia tepida*. Compared to the other two species, the pore depth (Pde) value in *Ammonia tepida* from Giordano et al. (2019) is shown to be much higher. Moreover, it is observed that a porosity% (Po) value in *Ammonia tepida* is lower than in *Ammonia parkinsoniana* but higher than in *Ammonia* cf. *beccarii*. The porosity% (Po) shows a direct relationship with the surface roughness (R) in *Ammonia* cf. *beccarii* and *Ammonia parkinsoniana*. Along with PD, Pdi, and Po, parameters such as Pde and R provide better quantification of the morpho-parameters. Using AFM provides a better understanding of the morpho-species variation, which has been demonstrated here in the genus *Ammonia*. Comparatively, species-specific pore depth (Pde) has been reported here for the first time. Pore parameters that characterize the *Ammonia* species reflect the morphofunctional adaptation to micro-habitat. Pore patterns in benthic foraminifera are directly correlated to the different environmental parameters, especially surrounding oxygen conditions (Richirt et al., 2019b).

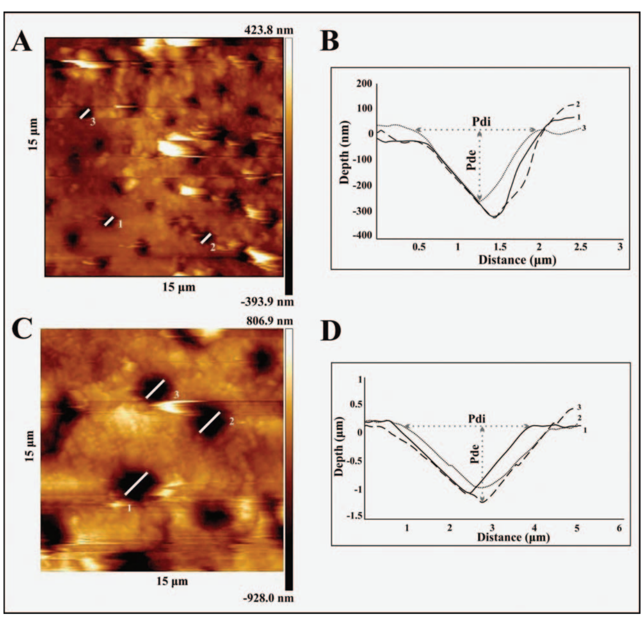

FIGURE 3. AFM measurement of pore diameter (Pdi) and pore depth (Pde) along a pore profile (scan area = 225 µm^2). **A** *Ammonia* cf. *beccarii.* **B** Pore profile showing Pdi and Pde of *Ammonia* cf. *beccarii.* **C** *Ammonia parkinsoniana.* **D** Pore profile showing Pdi and Pde of *Ammonia parkinsoniana.*

Ammonia beccarii in sandy substratum could be shallow endopelic (living inside the sediment) or epipelic (dwelling at the superficial sediment); *Ammonia tepida* is a free endopelic or epipelic species in the substratum with fine sediments (Debenay et al., 1998). As reported by Jorissen (1988) in the Adriatic Sea, the highest assemblage of *Ammonia beccarii* is associated with (>2%) of sand content and organic matter (0.8–1.1%) with intermediate values. Assemblages of *Ammonia parkinsoniana* inhabit the modern Po delta, where clayey and fine sandy substratum occur (D'Onofrio,1969; Fregni, 1980; Jorissen, 1988). Substratum with abundant organic matter (0.9–2.1%) and minimum sand fraction are preferable to *Ammonia tepida*, (Jorissen, 1988). The characteristic pore features of the species are correlative, ecophenotypic adaptations to the microhabitat preference. The relationship between the foraminiferal test and surface parameters with the microenvironmental condition is to be deciphered.

Understanding the species-specific characteristics of foraminifera can facilitate species comparison and identification, which play a vital role in paleoenvironmental and paleoceanographic reconstructions. This preliminary report provides a technique that can be applied to a larger dataset with proper statistical computation. It improves the technique of morphometric characterization and species identification in taxonomic classification.

CONCLUSIONS

The use of an Atomic Force Microscope has been demonstrated here for the first time in the species *Ammonia* cf.

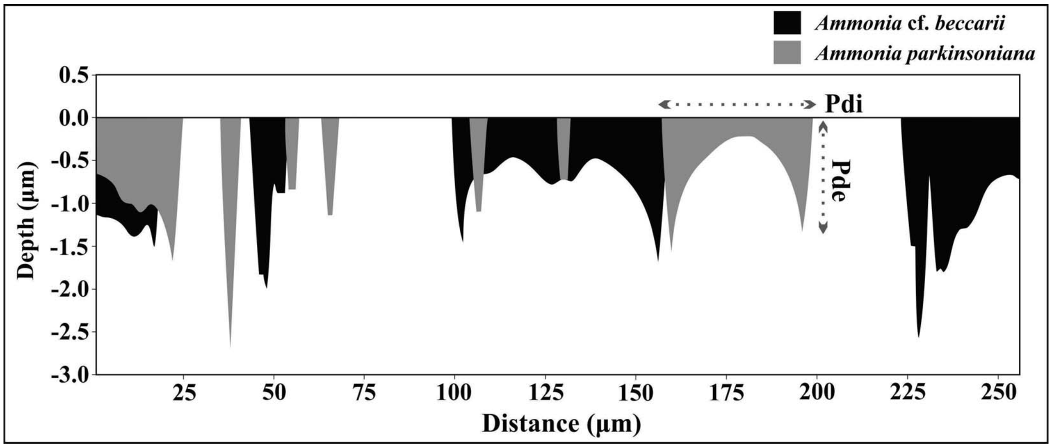

FIGURE 4. A comparative profile along a linear distance, with pore diameter (Pdi) and pore depth (Pde) of *Ammonia* cf. *beccarii* and *Ammonia parkinsoniana*.

beccarii and *Ammonia parkinsoniana* to measure various morpho-parameters on the foraminiferal surface. A comparison of different species of *Ammonia* is also shown, where pore depth is found to be species-specific. The accuracy in species characterization and identification is enhanced with the use of modern and improved tools in morphometric classification. AFM use in a larger dataset of foraminifera with different species would further explore the potential of three-dimensional measurement for accurate taxonomic classification.

ACKNOWLEDGMENTS

This research was funded by the Science and Engineering Research Board (SERB) Project (CRG/2021/005670), Government of India. The authors would like to acknowledge Professor Pratul Kumar Saraswati, Department of Earth Sciences, IIT Bombay and Dr. Abiral Tamang, Department of Physics, Jadavpur University for their valuable comments and support during the writing of this paper. We also thank the anonymous reviewers for their insightful comments.

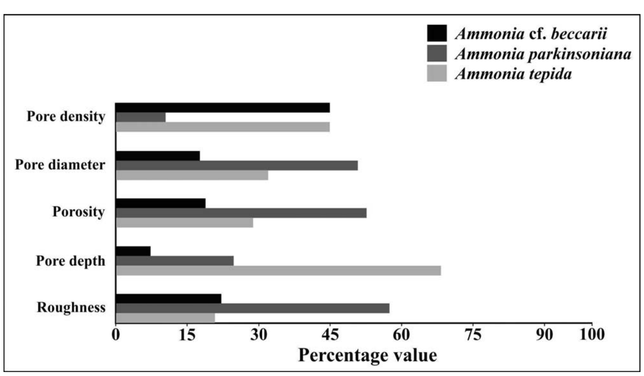

FIGURE 5. A comparative chart with different pore parameters of *Ammonia* cf. *beccarii*, *Ammonia parkinsoniana*, and *Ammonia tepida.* The data of *Ammonia tepida* from Giordano et al., 2019, tables 1–3, have been averaged.

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Received 13 September 2022
Accepted 23 January 2023