Heating Experiments on Benthic Foraminifera *Ammonia* sp. to Assess the Suitability of Amino Acid-Based Dating

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Abstract

Sub-milligram fossil biominerals, mainly benthic foraminifera, have been recently used to estimate the age of sediments determined from amino acid dating due to the minimum sample size requirement for reverse-phase high-performance liquid chromatography (RP-HPLC). This includes the collection of Ammonia sp., known as a key species occupying restricted lagoonal environments. However, an experiment has not been conducted to understand the racemization kinetics in order to examine the reliability of this species for amino acid racemization (AAR) geochronology. This research aims to determine the trend of the extent of racemization and the amino acid concentration, leading to the recommendation of Ammonia sp. shells as a dating specimen. The results exhibit a predictable pattern, demonstrating a consistently increasing extent of racemization after oven heating for up to 168 h without any indication of reversal pattern. The racemization rate differs from four selected amino acids reported in this study, where aspartic acid is the fastest, followed by glutamic acid, valine and isoleucine epimerization. Moreover, the consistent proportion of total amino acid and the decline of amino acid concentrations can be clearly observed following exponential decay. Therefore, this foraminifer can be used as an alternative specimen to establish amino acid-based geochronology, particularly in the depositional environment lacking other microfossils. Due to its confined habitat, amino acid-based dating of Ammonia sp. is considered applicable to understand the small environmental changes related to marginal marine successions.

Keywords: amino acid racemization, benthic foraminifera, Ammonia sp.

Introduction

Ammonia beccarii has been extensively recognized as an important species of benthic foraminifera in geological and environmental studies due to its abundance, restricted habitat and long duration within the geological timescale from Miocene to Holocene (Yassini and Jones, 1995; Debenay *et al.*, 1998; Hayward *et al.*, 2021). The occurrence of *Ammonia* sp., particularly *A. beccarii*, is commonly used to identify the changing habitat related to coastal and its associated depositional environments (Cann *et al.*, 1999). Cann *et al.* (2000), used the abundance of *A. beccarii* with *Elphidium articulatum* to understand the increasing influence of lagoonal and estuarine within the Murray River, southern Australia, by comparing it with different assemblage, particularly with normal inner-shelf marine foraminifera such as *Lamellodiscorbis dimidiatus* and *Elphidium crispum*. In the tropical maritime zone of Indonesia, Rositasari (2012) proposed applying *Ammonia* sp. as a quality indicator of the ocean by documenting the abnormal structure of the shells and their relative proportion with respect to the contaminated marine environments. Thus, the ecological, water quality, or environmental changes may be traced by performing geochronology of the initial or last occurrence of this species, such as radiocarbon dating (Cann *et al.*, 2000) or amino acid racemization (Murray-Wallace *et al.*, 2021).

Amino acid racemization (AAR) dating is considered one of the robust methods to estimate the age of fossil biominerals. The principle of racemization is closely associated with the asymmetric structure of amino acids as the building block of proteins. During the post-mortem period, amino acids within skeletal organisms, including foraminifera, undergo interconversion from a fully left-handed (L) amino acid structure to its right-handed (D) structure with the passage of time (Demarchi, 2020). It is noted that the higher concentration of D-amino acids with respect to L-amino acids corresponds to the older age of particular fossils, and thus, it provides potential use to assess the age of fossils (Wehmiller, 2013). Due to the slow process of racemization, the use of amino acid geochronology to calcium carbonate-based

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fossils has proliferated to date specimens within the Quaternary period beyond the limit of radiocarbon dating (Fallon and Murray-Wallace, 2020).

The technological advancement associated with minuscule sample requirements of reverse-phase high-performance liquid chromatography (Kaufman and Manley, 1998) provides an array of studies regarding the geochronological analysis of individual foraminifera. One of the successful amino acid dating studies by applying foraminifera, in this case, *Pulleniatina obliquiloculata*, was the positive trend of the extent of racemization with independent radiocarbon ages within the marine core of the northern Queensland margin (Hearty *et al.*, 2004). Hearty *et al.* (2004), noted the capability of amino acid dating to extrapolate the age beyond the limit of radiocarbon dating (~50 ka) with up to around 500 ka. Another application of AAR dating in foraminifera samples is the capability to understand both the timing and complex sources of carbonate sediments combined with other independent geochronological methods, mainly luminescence dating (Hidayat *et al.*, 2023). Concerning the genus *Ammonia,* the amino acid geochronology study by Ryan *et al.* (2020), has demonstrated the lack of reworking for this species in the modern sandflat facies close to the Murray River, southern Australia, as opposed to the beach sediments that contain older age foraminifera mainly from the Last Interglacial period (~125 ka). However, there is insufficient data associated with the kinetic behavior to determine whether or not this species is appropriate for AAR dating.

Due to the crucial attributes of *Ammonia* sp. in the geological and marine environmental aspects, this study aims to understand the trend of the extent of racemization and amino acid compositions by using isothermal heating experiments. Several studies have used this experiment to examine the reliability of certain species by analyzing some abnormal patterns. For example, the reversal extent of racemization (D/L values decrease with a more extended period of artificial heating) was shown in some mollusks such as *Ostrea*, particularly Aspartic acid (Asx), which complicates the interpretation of amino acid dating results (Kimber *et al.*, 1986). In this case, the experimental heating conducted in this study may provide a recommendation for selecting the most appropriate amino acids to be applied for age estimation.

Materials and Methods

The modern sediment used for this study was obtained from the sandflat facies previously collected by Ryan *et al.* (2020), at sediment surface below water level of Coorong Lagoon, southern Australia (Figure 1.). This sampling site is specifically included in the northern sector, having salinity of up to 50 PSU during winter based on the survey conducted in 2020 (Priestley *et al.*, 2022). From this modern sediment, as many as 45 specimens (adult, ~600 µm in size) of benthic foraminifera *Ammonia* sp. were hand-picked manually using a binocular microscope and sterile tweezers. Pristine and well-preserved foraminifera were prioritized in these heating experiments to minimize exogenous amino acid contamination (Figure 1.). Following this, collected *Ammonia* sp. individuals were prepared for AAR analysis based on treatment and analytical procedures from Kaufman and Manley (1998). A group of foraminifera was sonicated for 2 min within sterile vials to clean the outer surface from mainly clay sediments. As Blakemore *et al.* (2015) proposed, 2 M HCL etching was not used for delicate shells of this type of microfossil.

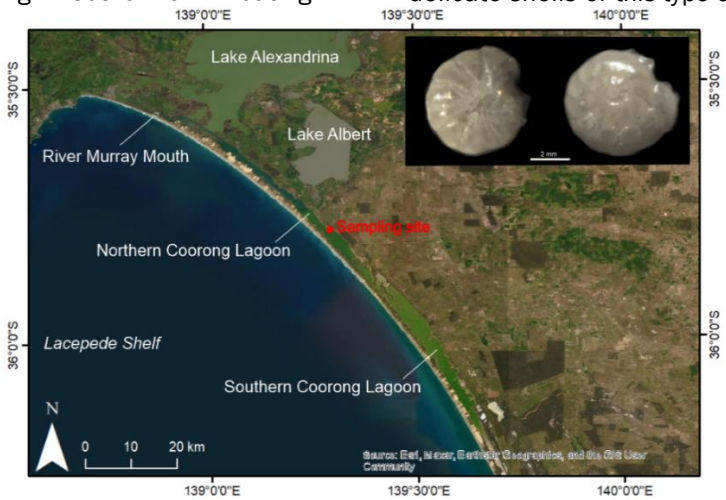

Figure 1. The Coorong Lagoon and sampling site for this research. The satellite map was gathered from ESRI (2009). Inset figure shows umbilical and spiral views of the representation of benthic foraminifera *Ammonia* specimens utilized for heating experiments.

Following this, a batch of *Ammonia* sp. (a total of 45 individuals) was separated into nine sterile vials related to the scheduled oven heating periods (0, 12, 24, 48, 72, 96, 120, 144 and 168 h). Each vial contains five individuals with a size of around 500 µm. The oven was continuously set to 110°C for the overall experiments. The designated vial was taken based on specific pyrolysis time and further treated with a 3% hydrogen peroxide (H_2O_2) solution for 2 h. Distilled water was used to clean the samples five times after removing the H_2O_2 solution. Foraminifera samples were air-dried at room temperature inside sterile plastic plates.

Once prepared, foraminifera shells were put into nine sterile micro-vials using a brush according to the pyrolysis time. A high-purity 6 M HCl solution (25 µL) was utilized to dissolve the shells before nitrogen gas flushing and oven hydrolysis at a temperature of 110°C for 22 h. Then, all samples within micro-vials were dried using a vacuum pump and further addition of L-*Homo*-Arginine solution as an internal standard to calculate relative amino acid concentrations. All micro vials were analyzed by RP-HPLC Agilent 1100 with a Hypersil C-18 BDS column. To ensure the integrity of measurement, we run interlaboratory standard ILC samples (Wehmiller, 2013) as well as four replicate analyses for each vials.

In this study, only total hydrolyzable amino acids (THAA) were obtained due to the small sample size of foraminifera. The extent of racemization (D/L value) is calculated based on the ratio between L- and D-amino acids. These D/L values were shown from three amino acids, Aspartic acid (Asx), Glutamic acid (Glx) and Valine (Val), with an additional extent of epimerization (Alle/Ile). Whereas the sum of concentration was calculated based on those four amino acids with the addition of Serine (Ser), Glycine (Gly), Alanine (Ala), and Phenylalanine (Phe). Since five foraminifera shells are transferred within one vial, the measured total amino acid concentrations should be divided to obtain the approximate value for individual foraminifera. Tables 1 and 2 show the results of the extent of racemization and concentrations of selected amino acids derived from heating experiments.

Results and Discussion

Extent of racemization

The increasing D/L values in benthic foraminifera *Ammonia* sp. can be observed from selected amino acids, particularly Asx. In 48 h, the extent of Asx racemization increases from 0.091 ±0.005 to 0.304±0.009 (Figure 2.). The rapid change in Asx D/L values is expected in fossil biominerals, including benthic foraminifera. For instance, Blakemore (2014), reported a similar trend of Asx D/L value in *L. dimidiatus* from roughly 0.062 to 0.336±0.002 during the first 48 h of 110°C of oven heating. Blakemore (2014), also mentioned that, for foraminifera *Elphidium crispum*, the exceptional incline of Asx D/L values is evident from 0.076 to 0.248±0.002 within the same period. However, after 48 h, the rate of Asx racemization appears slower, resulting in a gradual increase from 0.304±0.009 to 0.399±0.008 at the end of the experiments (168 h). This complicated trend of Asx is attributed to deamidation, where the initial process of racemization is controlled by faster-racemized aspargine rather than slower-racemized Aspartic acid (Geiger and Clarke, 1987). However, the measured D/L values of Asx follow a predictable trend by using a power equation with an R-squared of more than 0.93.

In addition, the more rapid racemization rate in Asx compared to other amino acids may be beneficial for determining age estimation within a short geological time scale. This trend was noted by Sloss *et al.* (2004), when using Asx of mollusk shells *Anadara trapezia* estimate the age of middle Holocene deposits since the typically rapid racemization rate provides better resolution after being calibrated by radiocarbon dating.

Table 1. The extents of racemization (D/L values) of *Ammonia* sp. according to heating experiments.

Heating time (hours)	UWGA code (replicates)	D/L values			
		Asx	Glx	Val	Alle/Ile
0	11443-A (4)	0.091±0.005	0.082±0.002	0.045±0.006	0.018±0.002
12	11443-B (4)	0.177±0.007	0.082±0.008	0.041±0.004	0.018±0.003
24	11443-C (4)	0.261±0.007	0.104±0.003	0.050±0.007	0.024±0.004
48	11443-D (4)	0.304±0.009	0.156±0.002	0.069±0.004	0.034±0.014
72	11443-E (4)	0.333±0.007	0.152±0.003	0.065±0.004	0.040±0.003
96	11443-F (4)	0.325±0.004	0.165±0.001	0.082±0.008	0.049±0.002
120	11443-G (4)	0.351±0.009	0.187±0.005	0.135±0.006	0.055±0.003
144	11443-H (4)	0.361±0.007	0.228±0.004	0.152±0.002	0.065±0.002
168	11443-I (4)	0.399±0.008	0.287±0.015	0.178±0.012	0.088±0.011

Table 2. The amino acid concentrations of *Ammonia* sp. according to heating experiments. Note that the concentrations are calculated from the average of individual foraminifera.

Heating time (hours)	UWGA code (replicates)	Amino acid concentrations (pmol.shell^{-1})			
		Asx	Glx	Val	Ile
0	11443-A (4)	238.9±6.4	160.4±2.8	127.7±4.7	74.2±1.4
12	11443-B (4)	175.0±4.2	137.5±3.4	97.6±3.2	64.9±0.9
24	11443-C (4)	171.6±11.9	114.1±3.0	90.8±7.8	55.5±2.1
48	11443-D (4)	122.8±4.3	82.8±3.5	65.2±3.2	27.9±0.2
72	11443-E (4)	110.7±3.5	81.8±2.5	60.1±1.6	36.1±1.0
96	11443-F (4)	95.2±1.3	65.9±0.6	51.8±1.7	30.2±0.5
120	11443-G (4)	82.6±3.0	50.0±2.9	34.0±1.9	17.1±1.1
144	11443-H (4)	87.5±2.1	68.8±2.3	44.9±1.5	28.6±0.6
168	11443-I (4)	67.5±1.0	45.3±0.7	33.4±1.0	20.5±0.4

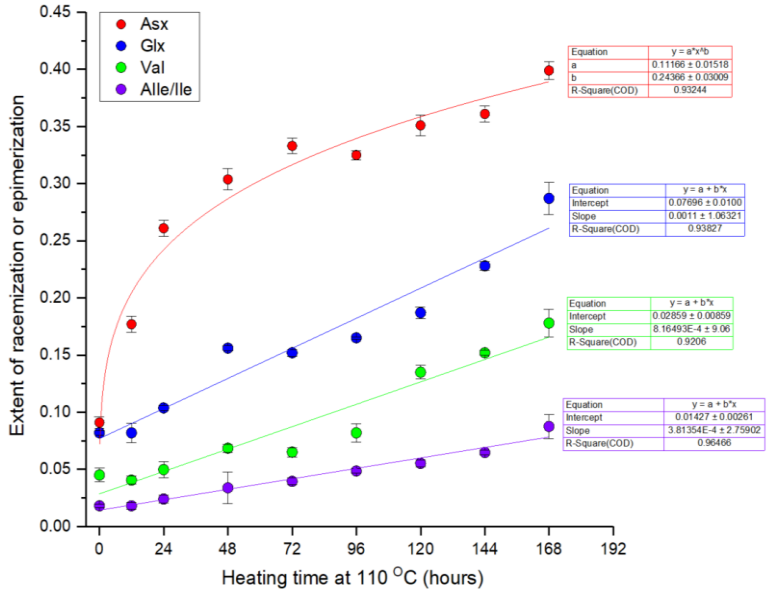

Figure 2. The extents of racemization of Asx, Glx, Val, and Alle/Ile in benthic foraminifera *Ammonia* sp. and the approximate linear and non-linear fittings.

The pattern of racemization from the other three amino acids is less complicated than Asx. Simple linear trends are sufficient to determine the gradual increase of racemization with an R-squared above 0.92 (Figure 2.). Clarke and Murray-Wallace (2006), also indicated that using linear equations is commonly suitable for the early phase of racemization, particularly below D/L values of 0.4. However, it is essential to note that these experiments were conducted within a short period (168 h) with measured D/L values of less than 0.3, except for Asx. Thus, the trend of each amino acid individual toward the racemic point (D/L values close to 1.0 or 1.3 for Alle/Ile) could not be further determined. In general, 768 h of heating time is required to compare the racemization rate of foraminifera samples (Blakemore, 2014).

Nevertheless, the results of this experiment are able to show the predicted trend of amino acids within *Ammonia* sp., which is a requirement of amino acid dating to provide reliable age estimation. Rapid reversal of D/L values, generally considered high uncertainty to establish amino acid dating (Kimber *et al.*, 1986), of four selected amino acids was not observed in this experiment. Moreover, the order of rate of racemization from these four amino acids can be determined from faster to slower: (1) Asx, (2) Glx, (3) Val and (4) Ile. This may be useful information for selecting appropriate amino acids depending on the age range of the deposits. In this case, Val and Ile are more suitable for assessing the geochronology of older deposits, particularly within the range up to the Pleistocene. The order of racemization rate

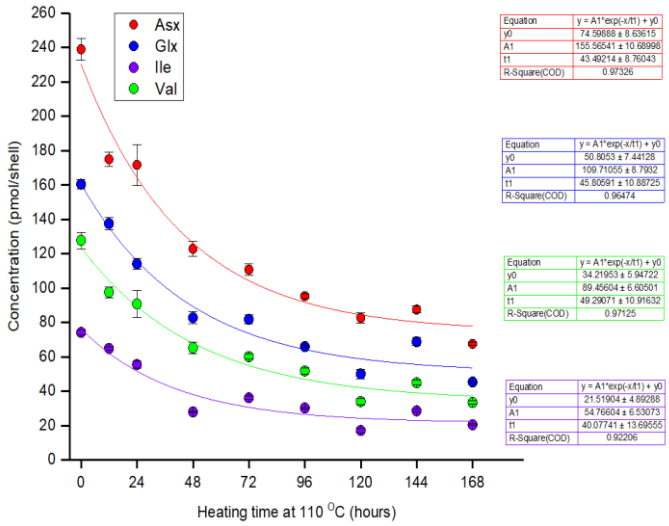

Figure 3. The amino acid concentration of selected amino acids in *Ammonia* sp. throughout pyrolysis time follows an exponential decay trend.

measured in *Ammonia* sp. is relatively similar to previous amino acid dating based on different foraminifera species.

In this case, Asx is generally considered to have a faster rate than Glx. For instance, Wheeler *et al*. (2021), showed the relationship between Asx and Glx within foraminifera *Neogloboquadrina pachyderma* (sinistral), where the extent of Asx racemization persistently higher than Glx throughout the deep marine succession at the Norwegian Sea. Similarly, using foraminifera *Elphidium advenum* subsp. *Macelliforme,* Murray-Wallace *et al.* (2021) also noted a higher degree of Asx racemization than Glx within the Upper Pleistocene (~75 ka) marine deposits of the southern Australia shelf. Due to better precision within the range of Pleistocene age, they opted to use Glx for establishing numerical age as opposed to Asx. Based on this, the selection of Asx and Glx as amino acid dating can be set according to the purpose of any given geochronological study, particularly in targeting different geological age ranges.

Amino acid abundance

The total amino acid concentration is the highest at the initial condition (no heating), up to 1251.5 pmol.shell^{-1}. In more detail, the Asx and Glx concentrations yielded 238.9 ± 6.4 pmol.shell^{-1} and 160.4±2.8 pmol.shell^{-1} (Figure 3.). It is noted that these values do not represent the 'true initial condition' as *Ammonia* shells were collected after their protein cessation. Thus, the total amino acid concentrations of the unheated sample measured in this experiment are expected to underestimate the 'initial' values. Nevertheless, this paper aims to see the trend throughout the pyrolysis experiment rather than determining the concentration of amino acids of a given species at the time after its death.

More rapid degradation of amino acid contents can certainly be recognized within 48 h of the 110°C heating period. Asx concentrations during this period are approximately half of the initial point, at 122.8 ± 4.3 pmol.shell^{-1} (Figure 3.). The degradation continues gradually up to the end of the experiment, from 122.8 to 67.5 pmol.shell^{-1}. Similarly, Glx content also decreases after 48 h, from 160.4 to 82.8 pmol.shell^{-1}, followed by relatively gradual changes toward 45.3 pmol.shell^{-1} within 168 h of the experiment (Figure 3.). This similar behavior of protein degradation concerning heating time also indicates the suitability of *Ammonia* sp. as an appropriate sample for amino acid dating. Up to 168 h, the selected amino acids also show a sufficient amount to be detected by the instrument, although the sample mass of foraminifera is relatively small. Moreover, the associated exponential decay line fitting for these amino acids displays more than 0.92, indicating a predictable pathway of protein degradation.

Amino acid proportions

The pattern of amino acid compositions is relatively consistent throughout the experiments, with no evidence of substantial changes in the proportion of individual amino acids. For instance, the percentage of Asx remains roughly between 19–23% (Figure 4.), although a slight increase is observed toward the end of the experiment. A similar

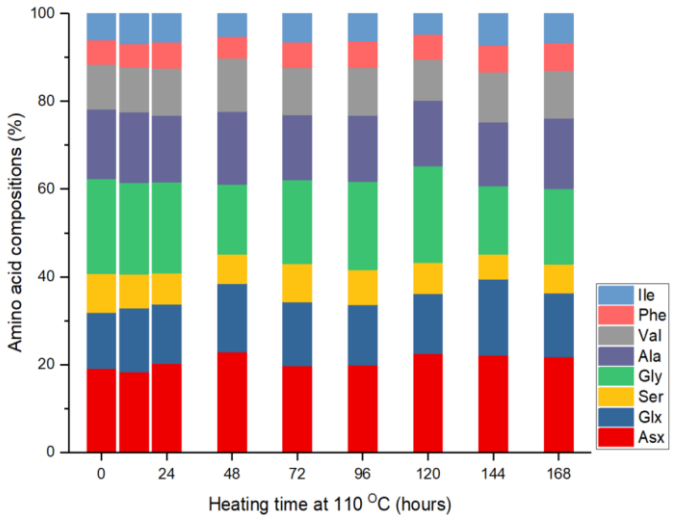

Figure 4. The relatively consistent trend of amino acid compositions in *Ammonia* sp. with respect to heating time.

configuration is evident in the proportion of Glx, ranging between 13–17% (Figure 4.). On the other hand, a small reduction can be seen in Gly percentage, particularly during the 144 and 168 h of oven heating.

The characteristics of increasing acidic amino acids, especially Asx, are somewhat similar to the other calcareous skeletons exposed to sodium hypochlorite (NaOCl) bleaching, which aims to obtain the intra-crystalline fraction of protein, such as *N. pachyderma* (Wheeler *et al.*, 2021) and mollusk *Phorcus lineatus* (Ortiz *et al.*, 2018). It may reflect that the heating process also removes the less resistant protein fraction, most likely intercrystalline matrices. It is suggested that the intercrystalline protein fraction of biominerals is lost during the long period of deposition and diagenesis, indicating that racemization extent is mainly dominated by the intra-crystalline, indigenous portion of peptides (Demarchi and Collins, 2015).

Bright and Kaufman (2011) noted that amino acid D/L values and concentrations are not statistically different in the intra-crystalline fraction of ostracoda *Candona* older than 1000 y old compared to its whole-shell, meaning that the intercrystalline matrices may have been removed due to protein diagenesis. Nevertheless, the relatively consistent compositions of amino acids within the period of experiments support the predictable trend of D/L values and reduction of amino acid concentrations, indicating the suitability of *Ammonia* sp. as a specimen for amino acid dating.

Conclusion

The heating experiment has been established to assess the suitability of amino acid geochronology using *Ammonia* sp. as a selection for sampling purposes. The pyrolysis results of isothermal 110°C for up to 168 h indicate a consistent increase in the extent of racemization without any evidence of reversal values. For this foraminifera species, Asx is considered the fast-racemizing amino acid, which can be used for dating younger geological age intervals, followed by slower racemizing amino acids (Glx, Val and Ile). The trend of decreasing concentration of those amino acids is also observed following exponential decay fit. Moreover, the percentages of amino acids appear to be relatively consistent toward the end of the experiments. These lines of evidence may indicate that *Ammonia* sp. may be a valuable alternative biomineral specimen for performing amino acid dating, particularly due to its restricted habitat within the coastal lagoon environments.

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