Chapter

3

Further evaluation of the trophic level estimation based on nitrogen isotopic composition of amino acids

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Abstract

Nitrogen isotopic composition ($\delta^{15}N$) of amino acids is a potentially useful as an alternative method for estimating the trophic level of organisms. The trophic level of organisms in food webs can be precisely estimated by comparing the high ^{15}N enrichment (+ 8.0‰) in glutamic acid and the little change (+ 0.4‰) in phenylalanine at each trophic level. Unlike the traditional method involving bulk isotope analysis, this amino acid method does not require characterization of the $\delta^{15}N$ values of primary producers for estimating the trophic level. In the present study, to further evaluate the applicability of the amino aicd method to a wide range of food web studies, (1) we investigate the isotopic signatures of amino acids (isotopic distribution in primary producers and ^{15}N enrichment factors) in terrestrial C3 and C4 plants and its consumer caterpillars, and (2) we apply this method to estimate the trophic level of various natural aquatic organisms. Although the isotopic distribution pattern differs considerably between aquatic photoautotrophs and terrestrial C3 and C4 plants, the ^{15}N enrichment factors in caterpillars are well consistent with those in the aquatic consumers reported in previous studies. We conclude that the trophic level ($TL_{Glu/Phe}$) can be estimated using the following equations: $TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4)/7.6 + 1$ for aquatic food webs, $TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + 8.4)/7.6 + 1$ for terrestrial C3 plant food webs, and $TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 0.4)/7.6 + 1$ for terrestrial C4 plant food webs. Moreover, on the basis of the present results and the previously published data, we demonstrate that the trophic level estimated by the amino acid method well reflects the actual food web structures in natural environments.

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INTRODUCTION

Stable isotope analysis of bulk organic materials has been employed in a number of ecological studies for investigating food web structures (Fry 2006). In particular, the nitrogen isotopic composition ($\delta^{15}N$) in bulk organisms and their tissues has been widely used to estimate the trophic levels of organisms and to study the nitrogen flow through food webs (*e.g.*, Hobson and Welch 1992; Yoshii et al. 1999; Ogawa et al. 2001). This "bulk method" is based on the empirical observation that the $\delta^{15}N$ value obtained for bulk organisms and their tissues tends to increase by approximately 3.4‰ with each trophic level (DeNiro and Epstein 1981; Minagawa and Wada 1984; Post 2002). However, the bulk method has several drawbacks. First, the ^{15}N enrichment factor, i.e., the increase of approximately 3.4‰ with each trophic level, varies for different samples (DeNiro and Epstein 1981; Vander Zanden and Rasmussen 2001; McCutchan Jr et al. 2003). This causes serious errors in the estimation of the trophic level. DeNiro and Epstein (1981) reported a large variation in the ^{15}N enrichment factor (-0.5 to $+9.2$‰) obtained for different animals, including insects and mammals. Second, it is necessary to characterize the $\delta^{15}N$ values corresponding to the primary producers when estimating the trophic level; however, this characterization is difficult in many cases. For example, spatial and temporal variabilities are observed in the $\delta^{15}N$ values corresponding to primary producers in the aquatic environment, such as cyanobacteria and algae (more than 10‰ in some cases; this corresponds to a value thrice the ^{15}N enrichment factor.). This is probably because of the assimilation of various nitrogen sources (*i.e.*, N_2, NO_3^-, NH_4^+) and the short life of the primary producers (Bronk and Glibert 1993; Rolff 2000; Dore et al. 2002; York et al. 2007). Therefore, primary producers collected from only a snapshot of natural environments do not always represent the realistic (or mean) $\delta^{15}N$ values of the primary producers in a food web (*e.g.*, O'Reilly et al. 2002).

Several recent studies have suggested that the nitrogen isotopic composition of amino acids is a useful alternative for estimating the trophic levels of various organisms (McClelland and Montoya 2002; Chikaraishi et al. 2007; McCarthy et al. 2007; Popp et al. 2007; Chikaraishi et al. 2009). It has been proposed that the trophic level of various organisms in a food web can be precisely estimated by comparing the large and small ^{15}N enrichment values in glutamic acid ($+8.0$‰) and phenylalanine ($+0.4$‰), respectively, at each trophic level (Fig. 1). This finding indicates a difference in the isotopic fractionations between the two amino acids during metabolic processes. In the case of glutamic acid, a significant degree of isotopic fractionation is observed during transamination because of the cleavage of the carbon-nitrogen bond; however, the $\delta^{15}N$ values of phenylalanine show only a slight change, as bonds involving nitrogen atoms are neither formed nor cleaved during the dominant process (Fig. 2). In fact, as demonstrated in Chikaraishi et al. (2009), the trophic

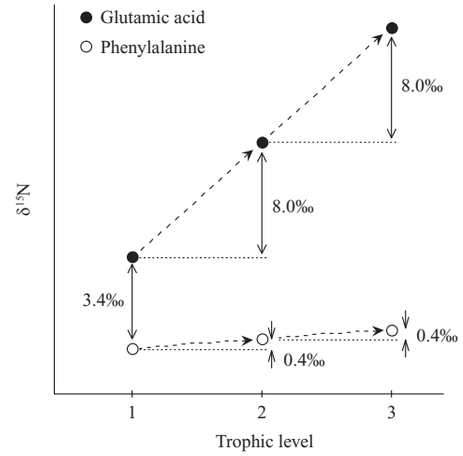

Fig. 1. Schematic illustration of the relationship between the nitrogen isotopic composition of amino acids (glutamic acid and phenylalanine) and trophic level in the aquatic food web (after Chikaraishi et al., 2009).

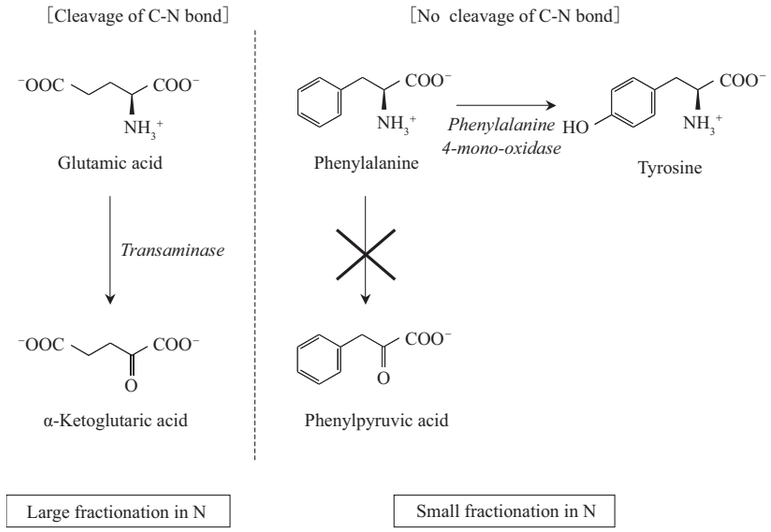

Fig. 2. Nitrogen isotopic fractionation during amino acid metabolisms in animals (after Chikaraishi et al., 2007).

levels of aquatic organisms are estimated with a small error ($1\sigma = 0.12$) by employing the following equation:

$$\text{Trophic level } (TL_{Glu/Phe}) = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4)/7.6 + 1 \quad (1)$$

where the value 3.4 is the isotopic difference between glutamic acid and phenylalanine in primary producers. The major advantage of this "amino acid method" is that the trophic level is estimated on the basis of the $\delta^{15}N$ values of two amino acids from a single organism. Consequently, unlike in the bulk method, it is not necessary for characterizing the $\delta^{15}N$ values of primary producers for estimation of the trophic level (McClelland and Montoya 2002; Chikaraishi et al. 2007, 2009). Further, because of the large value of the denominator (7.6‰) in equation (1), the error in the estimation of the trophic level is reduced. For this reason, nitrogen isotope analysis of amino acids has been used in recent ecological studies for elucidating the trophic level of various organisms (Pakhomov et al. 2004; McCarthy et al. 2007; Popp et al. 2007; Kashiyama et al. 2008).

However, the amino acid method is constructed to the experimental observations only from aquatic organisms in the previous studies (McClelland and Montoya 2002; Chikaraishi et al. 2007, 2009). It is uncertain whether equation (1) can be directly applied to food webs in terrestrial environments. Therefore, in the present study, we investigate the isotopic signatures of amino acids (*i.e.*, the isotopic distribution in primary producers and the ^{15}N enrichment factor at each trophic level) in terrestrial C3 and C4 plants and its consumer caterpillars. Moreover, we apply the amino acid method to various natural aquatic organisms (macroalgae, gastropods, and sharks in marine environments and phytoplankton, zooplankton, shrimp, and fish in freshwater environments). From the obtained results and previously published data, we confirm that the trophic level estimated by the amino acid method reflects the actual structure of food webs in natural environments.

Samples and Methods

We first examined the nitrogen isotopic composition of amino acids in 24 terrestrial organisms: 17 C3 plants, 5 C4 plants, and 2 herbivores (Tables 1 and 2). Terrestrial C3 plants *Castanea crenata* and *Brassica oleracea* were collected from a farm near Yokohama, Japan (35°08′N, 139°07′E) in November 2008. The herbivores (caterpillars) *Pieris rapae* were also collected from *B. oleracea* leaves during this period (*P. rapae* specifically feeds on *B. oleracea*). Other terrestrial C3 and C4 plants were collected from Japan and Thailand during 1998–2001; detailed information can be obtained from Chikaraishi et al. (2003). Secondly, we examind the nitrogen isotopic composition of the amino acids in several natural aquatic organisms, including macroalgae, gastropods, and sharks in marine environments and phytoplankton, zooplankton, shrimp, and fish in freshwater environments. The macroalga *Undaria pinnatifida* and gastropod *Cellana toreuma* were collected from the seacoast near Yokohama, Japan (35°08′N, 139°07′E) in May 2006 and

Table 1 Nitrogen isotopic composition of amino acids in terrestrial plants.

Sample	Type	Tissue	$\delta^{15}N$ (‰, relative to Air)										$TL_{Glu/Phe}$[a]
			Bulk	Ala	Gly	Val	Leu	Ile	Pro	Ser	Met	Glu	Phe	
C3 plants														
Acer argutum (#1)	Tree	Leaf	−2.7	−2.1	−12.9	−2.2	2.6	n.d.	5.0	−7.7	n.d.	1.0	6.2	1.4
Acer argutum (#2)	Tree	Leaf	−2.7	−1.7	−13.4	−4.9	−5.1	−6.6	5.4	−9.4	−11.7	−0.5	8.5	0.9
Acer carpinifolium (#1)	Tree	Leaf	−2.8	0.0	−12.4	−1.2	−2.1	−4.4	3.0	−4.5	−11.6	0.8	8.6	1.1
Acer carpinifolium (#2)	Tree	Leaf	−2.4	−3.4	−12.6	n.d.	−6.4	n.d.	5.8	−12.4	−12.3	−0.6	7.3	1.1
Artemisia princeps	Grass	Leaf	2.2	5.9	−4.1	6.9	0.1	3.2	10.2	−5.5	−3.7	8.7	18.7	0.8
Benthamidia japonica (#1)	Tree	Leaf	−2.0	−0.7	−10.8	−0.7	−2.4	−3.8	1.5	−4.7	−12.0	1.2	9.0	1.1
Benthamidia japonica (#2)	Tree	Leaf	−3.2	−0.4	−16.2	−0.6	−0.9	0.3	4.9	−10.0	n.d.	−0.4	9.5	0.8
Brassica oleracea	Grass	Leaf	n.d.	0.2	−6.7	5.1	3.8	3.9	9.5	1.0	0.8	5.7	13.1	1.1
Castanea crenata (#1)	Tree	Leaf	n.d.	−2.1	−12.7	−0.3	0.5	1.6	4.6	−4.2	n.d.	1.5	10.1	1.0
Castanea crenata (#2)	Tree	Nut	n.d.	n.d.	−14.8	n.d.	0.1	n.d.	2.9	−7.2	n.d.	0.8	8.3	1.1
Oryza sativa	Grass	Leaf	n.d.	−10.7	−9.6	n.d.	−11.1	−11.9	−1.7	−5.6	n.d.	−9.9	−1.0	0.9
Plantago asiatica	Grass	Leaf	−3.2	5.2	−10.3	2.0	−2.2	0.0	1.9	−8.7	−7.3	2.6	11.1	1.0
Prunus jamasakura	Tree	Leaf	3.1	7.9	−2.4	8.1	5.1	7.3	12.0	−4.4	−10.3	9.1	16.3	1.2
Quercus acutissima	Tree	Leaf	−3.7	5.0	1.8	4.4	0.4	2.0	8.4	−2.2	n.d.	4.5	14.0	0.8
Quercus dentata	Tree	Leaf	−6.0	−3.0	−8.0	−1.8	−7.5	−5.9	0.1	−9.2	n.d.	−2.5	4.9	1.1
Quercus mongolica	Tree	Leaf	7.4	n.d.	−2.1	n.d.	4.6	0.4	11.2	1.2	−0.3	9.7	17.0	1.2
Taraxacum officinale	Grass	Leaf	−1.8	2.6	−6.3	2.6	−4.9	−1.3	12.1	−8.5	−5.1	1.4	13.5	0.5
C4 plants														
Miscanthus sinensis	Grass	Leaf	−8.0	−10.0	−9.5	n.d.	−14.2	−11.4	−4.4	n.d.	n.d.	−6.3	−6.9	1.0
Saccharum officinarum (#1)	Grass	Leaf	−4.2	−6.6	−5.8	n.d.	−9.3	n.d.	−0.9	−4.8	n.d.	−1.1	−2.2	1.1
Saccharum officinarum (#2)	Grass	Leaf	4.3	7.9	6.8	11.5	6.1	6.8	10.0	4.2	−0.2	8.0	7.3	1.0
Sorghum bicolor	Grass	Leaf	6.7	11.1	9.1	12.0	9.8	7.8	13.3	1.0	11.5	11.9	14.7	0.6
Zea mays (#1)	Grass	Leaf	2.6	n.d.	1.1	n.d.	1.3	n.d.	3.8	−0.7	−4.7	6.9	4.0	1.3
Zea mays (#2)	Grass	Leaf	2.8	n.d.	n.d.	n.d.	0.0	n.d.	3.6	−12.5	5.3	5.3	4.9	1.0
Zoysia japonica	Grass	Leaf	2.5	1.9	1.9	n.d.	2.2	3.0	6.7	1.9	−0.4	6.4	6.1	1.0

[a] Trophic level calculated by the amino acid method using the following equation:
C3 plants: $TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + 8.4)/7.6 + 1$; see text
C4 plants: $TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 0.4)/7.6 + 1$; see text

Table 2 Nitrogen isotopic composition of amino acids in caterpillars.

Sample	Food source	δ^{15}N (‰, relative to Air)											$TL_{Glu/Phe}$[a]
		Bulk	Ala	Gly	Val	Leu	Ile	Pro	Ser	Met	Glu	Phe	
Pieris rapae (#1)	*B. oleracea*[b]	n.d.	6.6	−0.4	8.2	7.0	8.9	16.3	3.7	1.6	13.0	13.4	2.0
Pieris rapae (#2)	*B. oleracea*[b]	n.d.	5.3	−2.7	7.4	6.5	8.5	14.3	2.3	1.0	14.6	13.6	2.2

[a] Trophic level calculated by the amino acid method using the following equation: : $TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + 8.4)/7.6 + 1$; see text

[b] δ^{15}N values of amino acids in the food source (*B. oleracea*) are listed in Table 1.

February 2001, respectively. The shark *Squalus* sp. was obtained from the offshore area in Okinawa in March 2008. Phytoplankton (an admixture of *Volvox* sp., *Eudorina* sp., and *Chlamydomonas* sp.) and the zooplankton *Daphnia* sp. were collected from Lake Sagami (35°36′N, 139°11′E) in October 1999. The shrimp *Caridina multidentata* was collected from a pond near Yokohama, Japan (35°08′N, 139°07′E) in December 2008. The fish *Micropterus salmoides* and *Zacco platypus* were collected from Lake Biwa in June 1995. The fish *Gymnogobius isaza* was collected from the Lake Biwa during 1916−1982 (Ogawa et al. 2001). These samples were cleaned with distilled water to remove contaminants and stored at −20°C. Most of the samples were freeze-dried and crushed to a fine powder before analysis, and the freshwater fish samples (*M. salmodes*, *G. isaza*, and *Z. platypus*) were stored in formalin: there was no effect on the nitrogen isotopic composition of the amino acids (Ogawa et al., unpublished data). Small pieces of the muscular tissues of the fish samples were used for our analysis. The nitrogen isotopic composition of the bulk sample materials was determined using a Thermo Fisher Scientific Flash EA (1112EA) coupled to a Delta^{plus}XP IRMS *via* a ConFlo III interface. (Ohkouchi et al., 2005; Ogawa et al., 2010).

The above samples were used for the compound-specific nitrogen isotope analysis of amino acids after HCl hydrolysis and *N*-pivaloyl/isopropyl ester (Pv/iPr) derivatization, according to the methods described in Chikaraishi et al. (2007). In brief, each sample was hydrolyzed using 12 M HCl at 100°C, and the hydrolysate was washed with *n*-hexane/dichloromethane (6: 5, v/v) for the removal of hydrophobic constituents such as lipids. After derivatization with thionyl chloride/2-propanol (1: 4, v/v) and then with pivaloyl chloride/dichloromethane (1: 4, v/v), the Pv/iPr derivatives of the amino acids were extracted with *n*-hexane/dichloromethane (6: 5, v/v). The nitrogen isotopic composition of individual amino acids was determined by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) using an Agilent Technologies 6890N GC coupled to a Thermo Fisher Scientific Delta^{plus}XP IRMS *via* a GC-C/TC III interface (Hayes et al. 1990; Brand et al. 1994; Merritt and Hayes 1994). The analytical conditions used in GC/C/IRMS are described in detail in Chikaraishi et al. (2010).

The nitrogen isotopic composition is expressed as conventional δ notation against atmospheric N_2 (Air). To confirm the reproducibility of the isotope measurements, standard mixtures of eight amino acids (alanine, glycine, valine, leucine, asparatic acid, serine,

glutamic acid, and phenylalanine) whose δ^{15}N values were known were analyzed every four or five GC/C/IRMS runs. The analytical error (1σ) obtained for the standards was always better than 0.5‰ when the minimum amount of sample used was 30 ngN. The δ^{15}N values of the following 10 amino acids were determined: alanine, glycine, valine, leucine, isoleucine, proline, serine, methionine, glutamic acid, and phenylalanine. These amino acids were chosen because they always gave well-separated peaks with baseline resolution in the chromatogram (Metges et al. 1996, Chikaraishi et al., 2010). Since glutamine was converted to glutamic acid during acid hydrolysis, the α-amino group of glutamine contributed to the δ^{15}N value obtained for glutamic acid. The isotopic compositions of other amino acids were not determined in our experiment because a portion of aspartic acid co-eluted with threonine on the chromatogram, while arginine, cysteine, hystidine, lysine, tyrosine, and tryptophan were not detected in the chromatogram, probably because they were decomposed or recovered in low yields during the procedures.

Results and Discussion

Distribution of δ^{15}N among amino acids in terrestrial C3 and C4 plants

The δ^{15}N values of the amino acids isolated from terrestrial C3 and C4 plants (Table 1) varied over a wide range (from −16.2 to +18.7‰). These values were indicative of the assimilation of isotopically variable nitrogen and the unique isotopic fractionation of nitrogen associated with amino acid biosynthesis, as in the case of aquatic photoautotrophs (McClelland and Montoya 2002; Chikaraishi et al. 2007). The difference between the δ^{15}N values of the amino acids and the δ^{15}N value of phenylalanine ($\delta^{15}N_{x/Phe}$) is shown in Fig. 3, along with previously published data for aquatic photoautotrophs (McClelland and Montoya 2002; Chikaraishi et al. 2007; 2009). Although the $\delta^{15}N_{x/Phe}$ values varied over a broad range (from −26.6 to −0.7‰ for C3 plants and from −17.4 to +4.2‰ for C4 plants), the $\delta^{15}N_{x/Phe}$ pattern was almost similar within a single plant taxon, even for different types of plants (*i.e.*, trees and grasses) and for different tissues (leaf *vs* nut) of a given plant. In particular, the $\delta^{15}N_{x/Phe}$ values of glutamic acid present in plants within a single taxon were almost similar with very small variations (1σ = 1.6‰ for C3 plants and 1.7‰ for C4 plants) (mean values and variations (1σ) are summarized in Table 3). This implied that the δ^{15}N value of glutamic acid could be useful for estimating the trophic levels in terrestrial food webs.

However, the $\delta^{15}N_{x/Phe}$ pattern in C3 plants is clearly distinct from that in C4 plants and aquatic photoautotrophs (Fig. 3). In the case of C3 plants, all amino acids are much depleted in ^{15}N relative to phenylalanine. In contrast, in the case of C4 plants, alanine, glycine, leucine, isoleucine, serine and methionine are relatively less depleted in ^{15}N relative to phenylalanine, whereas valine, proline and glutamic acid have similar δ^{15}N values to phenylalanine. These results clearly indicate that equation (1), which is used for estimating the trophic level of aquatic organisms, cannot be directly extended to food webs in terrestrial environments. The isotopic difference between glutamic acid and phenylalanine (−3.4 for

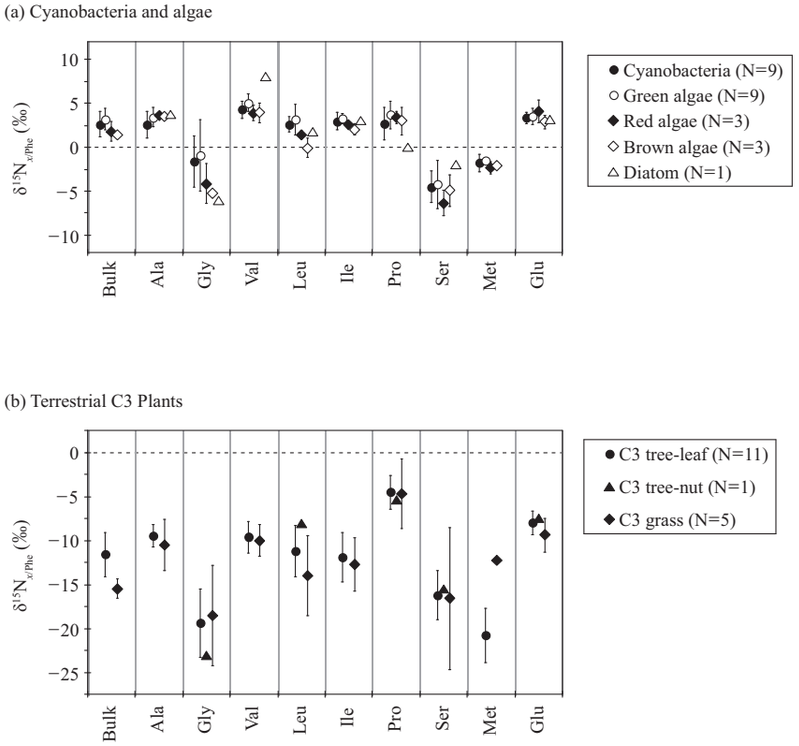

Fig. 3. Nitrogen isotopic composition of amino acids with respect to that of phenylalanine ($\delta^{15}N_{x/Phe}$) for primary producers: (a) cyanobacteria and algae (McClelland and Montoya 2002; Chikaraishi et al. 2007; 2009) and (b) terrestrial C3 and C4 plants (present study). Symbols and bars represent the mean and variation (1σ) for a single taxon of primary producers (types and tissues are identified for C3 plants), respectively.

Table 3 Summary of the isotope difference between amino acids and phenylalanine in primary producers, ^{15}N-enrichment factor at each trophic level, and the corresponding variability (1σ)

	Isotope difference between amino acids and phenylalanine						^{15}N-enrichment factor[b]	
	Cyanobacteria and algae[a]		Terretarial C3 plants		Terrestrial C4 plants			
	Mean	1σ	Mean	1 σ	Mean	1σ	Mean	1σ
Bulk	2.6	1.3	−12.5	2.8	−3.1	2.4	2.1	1.3
Alanine	3.2	1.2	−9.8	2.0	−2.9	2.1	6.0	1.9
Glycine	−2.3	3.4	−19.3	4.3	−3.2	1.7	4.0	3.5
Valine	4.6	1.2	−9.7	1.8	0.8	4.9	4.6	1.8
Leucine	2.3	1.6	−11.8	3.6	−4.6	2.2	4.4	1.9
Isoleuicine	2.9	0.8	−12.1	2.8	−3.7	2.7	4.8	1.5
Proline	3.1	1.7	−4.6	2.5	0.6	1.7	6.1	1.5
Serine	−4.6	2.2	−16.3	4.6	−7.6	6.3	3.3	2.8
Methionine	−2.0	0.6	−19.7	3.7	−5.1	3.7	0.5	0.5
Glutamic acid	3.4	0.9	−8.4	1.6	0.4	1.7	8.0	1.1
Phenylalanine	–	–	–	–	–	–	0.4	0.4

[a] Data from Chikaraishi et al. (2009)

[b] Mean values for zooplankton, fish, gastropod, and caterpillar reported in previous studies (McClelland and Montoya 2003; Chikaraishi et al. 2007, 2009) and in the present study.

aquatic food webs) should be replaced with +8.4 for C3 plants or −0.4 for C4 plant food webs.

Theoretically, the isotopic fractionation of biomolecules mainly depends on the kinetic isotope effect and flow on enzymatic reactions. Therefore, it is clear that at least within a single plant taxon, amino acids are biosynthesized via common pathways with the almost same kinetic isotope effect and flow in enzymatic transaminations. However, the $\delta^{15}N_{x/Phe}$ values differ markedly among terrestrial C3 and C4 plants and aquatic photoautotrophs (Fig. 3), implying the distinctly different mechanisms in these producers. The exact cause of the difference in the $\delta^{15}N_{x/Phe}$ values is presently unknown; however, it is speculated that this difference reflects the distinct kinetic isotopic effect or the flow rate associated with enzymatic transamination reactions in these producers or the significant difference in the mechanism of assimilation of inorganic nitrogen or biosynthetic pathways of amino acids among these producers.

^{15}N Enrichment factor of amino acids at each trophic level

The $\delta^{15}N$ values of the amino acids present in *P. rapae* caterpillars also vary widely, i.e., from −2.7 to +16.3‰ (Table 2); most of the amino acids such as alanine, valine, leucine, isoleucine, proline, and glutamic acid are enriched in ^{15}N (up to 8.8‰ for glutamic acid) relative to the corresponding amino acids in the food source *B. oleracea*, whereas methionine and phenylalanine show little difference from those in *B. oleracea* (Fig. 4). However, this ^{15}N enrichment pattern at a given trophic level for a plant leaf-caterpillar combination is very similar or consistent with that for phytoplankton-zooplankton,

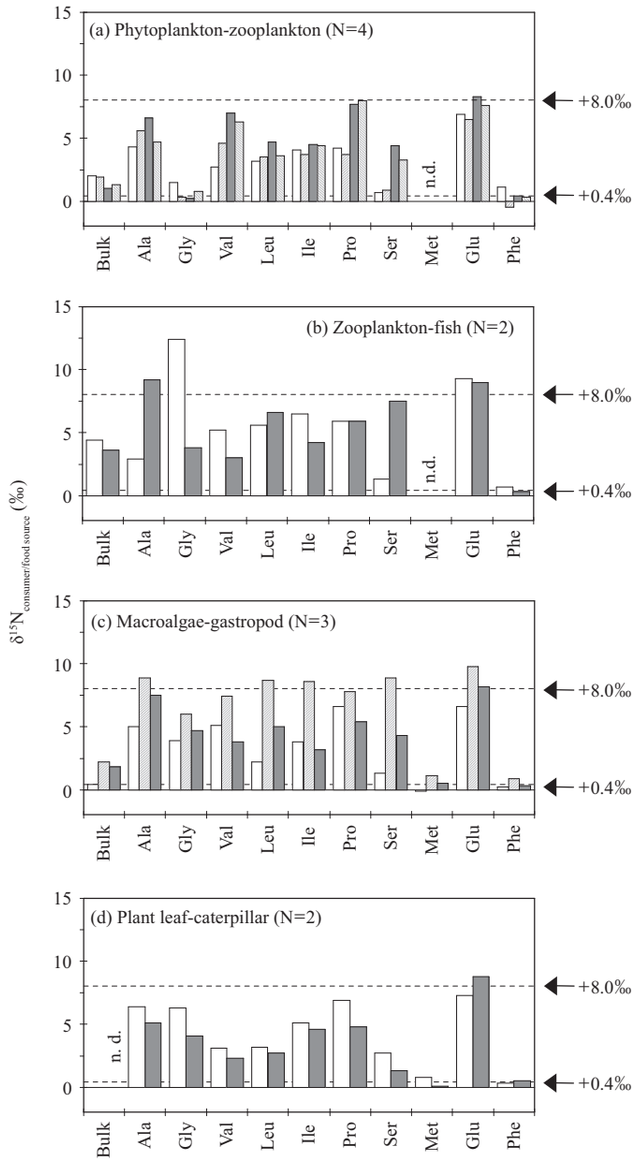

Fig. 4. ^{15}N enrichment with trophic level ($\delta^{15}N_{consumer/food\ source}$) for four food-consumer combinations: (a) phytoplankton-zooplankton, (b) zooplankton-fish and (c) macroalgae-gastropod (McClelland and Montoya 2002; Chikaraishi et al. 2007; 2009), and (d) plant leaf-caterpillar (present study) Different pillars represent different samples.

zooplankton-fish, and macroalgae-gastropod combinations reported in previous studies (Fig. 4). This implies that in all food-consumer combinations, the $\delta^{15}N$ values of the consumer amino acids mainly reflect the same isotopic fractionation processes occurring during amino acid metabolism (Fig. 2, see Chikaraishi et al. 2007). From these results, we infer that the ^{15}N enrichment factor of amino acids at each trophic level can be directly applied to a wide range of consumers, including organisms in terrestrial food webs (mean ^{15}N enrichment factors and variations (1σ) are summarized in Table 3).

Trophic level estimation based on the nitrogen isotopic composition of amino acids

As described above and summarized in Table 3, the isotopic difference between glutamic acid and phenylalanine ($\delta^{15}N_{Glu/Phe}$) is markedly different among aquatic photoautotrophs (3.4 ± 0.9‰), terrestrial C3 (-8.4 ± 1.6‰) plants, and C4 plants (0.4 ± 1.7‰), indicating that the corresponding value in equation (1) (*i.e.*, -3.4) for aquatic food webs should be replaced with $+8.4$ for C3 plant webs or with -0.4 for C4 plant food webs. On the other hand, the ^{15}N enrichments factors of zooplankton, gastropods, and fish in aquatic food webs (stated in previous studies) and caterpillars in terrestrial food webs (stated in the present study) are very similar (or consistent); this indicates that the ^{15}N enrichment factor can be used in the study of various organisms in aquatic as well as terrestrial food webs. Therefore, we conclude that the trophic level for terrestrial C3 and C4 plant food webs can be estimated by employing equations (2) and (3), respectively.

$$TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + 8.4)/7.6 + 1 \quad (2)$$

$$TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 0.4)/7.6 + 1 \quad (3)$$

When the above equations are applied to the samples with known trophic levels listed in Tables 1 and 2 (terrestrial plants: 1.0, caterpillars: 2.0), the obtained values include a small error ($1\sigma = 0.20$). Although the error obtained in the case of terrestrial food webs is larger than that observed in the case of aquatic food webs ($1\sigma = 0.12$, see Chikaraishi et al. 2009), it is sufficient ($1\sigma = 0.20$) for identifying the trophic level of terrestrial plants and caterpillars. The relatively large error in the results obtained for terrestrial food webs is probably due to the fact that the variation in the $\delta^{15}N_{Glu/Phe}$ values of terrestrial C3 ($1\sigma = 1.6$‰) and C4 plants ($1\sigma = 1.7$‰) is larger that in the $\delta^{15}N_{Glu/Phe}$ values of aquatic photoautotrophs ($1\sigma = 0.9$‰). This result may be partly explained on the basis of the large heterogeneity in the isotopic composition of nitrogen sources in terrestrial environments. In fact, it has been known that terrestrial plants grow with various nutrient sources and that their $\delta^{15}N$ values vary over a wide range (Werner et al., 2002).

Application of the amino acid method to natural food web studies

To further evaluate the applicability of the amino acid method to a wide range of food web studies, we estimate the $TL_{Glu/Phe}$ values of natural aquatic organisms, including macroalgae, gastropods, and sharks in marine environments and phytoplankton, zooplankton,

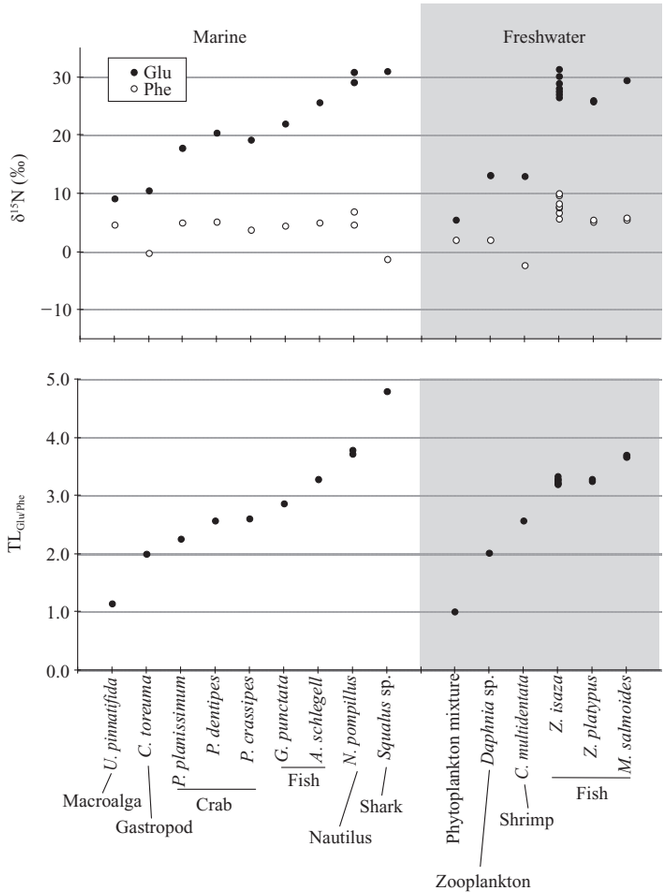

Fig. 5. $\delta^{15}N$ values of glutamic acid and phenylalanine and $TL_{Glu/Phe}$ values in natural organisms collected from natural marine, freshwater, and terrestrial environments. Data from previously published studies (Kashiyama et al. 2010; Chikaraishi et al. 2009) are also provided.

shrimp, and fish in freshwater environments. The nitrogen isotopic compositions of glutamic acid and phenylalanine and the obtained $TL_{Glu/Phe}$ values are summarized in Fig. 5, along with the previously published values for crab and fish (Chikaraishi et al. 2009) and nautilus (Kashiyama et al. 2010).

As mentioned above, the $\delta^{15}N$ values of aquatic primary producers vary because of the assimilation of various nitrogen sources and the utilization efficiencies of the sources, as well as the short life of the primary producers (*e.g.*, Rolff 2000; O'Reilly et al. 2002). Such

a variation is also seen in the $\delta^{15}N$ values of phenylalanine (which has little effect with trophic level), by 8.2‰ (from −1.3 to +6.9‰) for marine and 12.1‰ (from −2.3 to +10.1‰) for freshwater environments (Fig. 5).

However, as shown in Fig. 5, the $TL_{Glu/Phe}$ values for macroalga (1.1) and gastropods (2.0) in marine environments and those for phytoplankton (1.0) and zooplankton (2.0) in freshwater environments are consistent with the trophic levels of primary producers (trophic level = 1.0) and primary consumers (trophic level = 2.0), respectively. Also, the $TL_{Glu/Phe}$ values for crabs (2.3−2.6), fish (2.9−3.3), nautilus (3.7−3.8), and sharks (4.8) in marine environments and those for shrimps (2.6) and fish (3.2−3.7) in freshwater environments are considered to be adequate values for the trophic level of these omnivores and carnivores. Thus, we state that the $TL_{Glu/Phe}$ values reflect the actual trophic level in natural food webs in marine and freshwater environments.

Conclusions

The amino acid method can be used to estimate the trophic level from the $\delta^{15}N$ values of two amino acids (glutamic acid and phenylalanine) from a single organism. The error in the results obtained with this method ($1\sigma = 0.12$ for aquatic and $1\sigma = 0.20$ for terrestrial organisms) is smaller than that in the results obtained using the bulk method. In the present study, we investigate the isotopic signatures of amino acids in terrestrial C3 and C4 plants and their consumers (caterpillars). From the results, we conclude that the trophic level ($TL_{Glu/Phe}$) can be estimated by employing the following equations:

$$TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4)/7.6 + 1 \text{ (for aquatic food webs)}$$
$$TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + 8.4)/7.6 + 1 \text{ (for terrestrial C3 plant food webs)}$$
$$TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 0.4)/7.6 + 1 \text{ (for terrestrial C4 plant food webs)}$$

However, the difference in the equations used for determining $TL_{Glu/Phe}$ in aquatic and terrestrial C3 and C4 plant food webs leads to difficulties on the estimation of trophic level of organisms where stand on an admixture of two or three types of food webs. In such a case, additional information such as the carbon isotope signature is necessary for estimating the trophic level. Nevertheless, it is clear that the amino acid method will be a powerful alternative tool for elucidating the trophic level of organisms in ecological food web studies. An important advantage of this method is that characterization of the $\delta^{15}N$ values of the primary producers is not necessary; hence, estimation of the trophic levels of various organisms becomes easy, and one can gain a thorough understanding of the actual structures of food webs and the nitrogen flow in natural environments. Moreover, only nanomolar amount of nitrogen is required for the precise determination of the isotopic composition of a single amino acid by GC/C/IRMS. For example, we used very small amounts of the sample (dry weight: 0.1−1.0 mg) in the present study. This small sample size also facilitates the estimation of the trophic level for a wide range of organisms, including natural

microorganisms, growth layers in fish scales, and residual proteins in fossil bones.

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