Stable isotope fingerprinting: a novel method for identifying plant, fungal, or bacterial origins of amino acids

Thomas Larsen, D. Lee Taylor, Mary Beth Leigh, and Diane M. O'Brien[1]

Institute of Arctic Biology and Department of Biology and Wildlife, University of Alaska, Fairbanks, Alaska 99775-7000 USA

Abstract. Amino acids play an important role in ecology as essential nutrients for animals and as currencies in symbiotic associations. Here we present a new approach to tracing the origins of amino acids by identifying unique patterns of carbon isotope signatures generated by amino acid synthesis in plants, fungi, and bacteria ("^{13}C fingerprints"). We measured amino acid δ^{13}C from 10 C$_3$ plants, 13 fungi, and 10 bacteria collected and isolated from a boreal forest in interior Alaska, USA, using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Microorganisms were cultured under amino-acid-free conditions and identified based on DNA sequences. Bacteria, fungi, and plants generated consistent, unique ^{13}C fingerprints based on the more complex amino acids (five or more biosynthetic steps) that are classified as essential for animals. Linear discriminant analysis classified all samples correctly with >99% certainty and correctly classified nearly all insect samples from a previous study by diet. Our results suggest that ^{13}C fingerprints of amino acids could provide a powerful in situ assay of the biosynthetic sources of amino acids and a potential new tool for understanding nutritional linkages in food webs.

Key words: Alaska, USA; compound-specific stable isotope analysis; δ^{13}C; essential amino acids; eukaryotes; gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS); prokaryotes.

Introduction

Understanding nutritional linkages is a central but at times elusive goal of both organismal biology and ecosystem ecology. Amino acids, especially those that are essential for animals, are key nutrients shaping foraging behavior (O'Brien et al. 2003, Gietzen et al. 2007) and creating nutritional interdependencies, such as between phytophagous insects and their microbial endosymbionts (Baumann et al. 2006, Janson et al. 2008). Amino acid exchange may also occur in symbiotic associations between plants and fungi, a situation in which each partner has the ability to synthesize amino acids but fungi may also absorb amino acids from the substrate (Read and Perez-Moreno 2003, Brundrett 2004). A marker of amino acid biosynthetic origin would be a powerful tool for investigations of amino acid exchange in symbiotic and trophic relationships, as well as a way to monitor diet in organisms that are otherwise intractable for studies of foraging behavior such as soil microarthropods (Chahartaghi et al. 2005, Larsen et al. 2007). Here we present a new approach to tracing the origins of amino acids: using unique patterns of carbon isotope signatures generated by amino acid synthesis in plants, fungi, and bacteria (here termed a "^{13}C fingerprint").

Amino acids vary widely in isotope composition within organisms (Abelson and Hoering 1961, Macko et al. 1987, Hare et al. 1991, Fantle et al. 1999, Fogel and Tuross 1999, Abraham and Hesse 2003, McCarthy et al. 2004), due to isotopic discrimination by biosynthetic pathways (Hayes 1993, 2001). Organisms that share similar enzymes and pathway architecture should exhibit similar patterns of isotope fractionation; however, identifying patterns characteristic of particular lineages has been hampered by limited taxonomic sampling, differences among studies in the amino acids measured, and varying growth conditions (Hayes 2001). Recently amino acid δ^{13}C was surveyed in a large sample of microorganisms (Scott et al. 2006); these authors found that patterns of amino acid variation grouped organisms by their mode of carbon acquisition and intermediary carbon metabolism. Similarly, characteristic patterns of amino acid δ^{13}C have been shown to distinguish fungal lineages (Abraham and Hesse 2003).

We suggest that differences in amino acid metabolism among major prokaryotic and eukaryotic lineages could generate unique patterns of amino acid δ^{13}C. For example, plants and fungi have unique pathways for lysine synthesis (Hudson et al. 2005), and their pathways for leucine biosynthesis are compartmentalized differently (Hagelstein et al. 1997, Kohlhaw 2003). No studies have yet systematically characterized the ^{13}C fingerprints of amino acids from fungi, plants, and bacteria, to see whether they are consistent within and distinct between lineages and whether they could be used to identify the origin of amino acids important in trophic or symbiotic

relationships. The advantage of such a fingerprint for ecological studies is that organisms would generate them naturally; thus, it could provide a powerful tool for identifying amino acid sources in situ.

To investigate whether fungi, bacteria, and plants generate unique amino acid ^{13}C fingerprints, we cultured 10 or more specimens from each group on amino-acid-free media and analyzed their amino acid ^{13}C composition using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) (Silfer et al. 1991, Meier-Augenstein 1999). Most of the specimens were cultured from a boreal forest site near Fairbanks, Alaska, USA, and all plants had the C$_3$ photosynthetic pathway. We hypothesized that fungi, bacteria, and plants generate distinct, consistent isotope fingerprints that could be used to classify samples to each lineage. We expect the amino acids that are essential for animals to be particularly informative because these amino acids have longer and more complex biosynthetic pathways (five or more biosynthetic steps), providing more potential for lineage-specific isotope effects (Lehninger 1975, Stephanopoulos et al. 1998). Furthermore, because animals depend on diet (Robbins 1993) or microbial symbionts (Robbins 1993, Moran and Baumann 2000, Wernegreen 2002) for essential amino acids, these compounds are particularly well suited for investigating trophic and symbiotic relationships (Fantle et al. 1999, O'Brien et al. 2002, 2005). Thus, we make an operational distinction between essential and nonessential amino acids in this study even though the terms do not apply in a functionally relevant sense to plants, fungi, or bacteria.

MATERIALS AND METHODS

Specimens

A total of 33 specimens, 13 fungi, 10 bacteria, and 10 plants were included in this study (Table 1). The majority of these specimens, 21 out of 31, were collected or isolated from a boreal forest site in interior Alaska with permafrost dominated by black spruce (64°52′19″ N, 147°50′59″ W). We also included specimens of fungi and bacteria from existing laboratory cultures at the University of Alaska Fairbanks (UAF), as well as domesticated plants grown in the UAF West Ridge greenhouse.

The soil samples from which we isolated fungi and bacteria were collected in April 2007. We selected our fungal and microbial specimens according to two criteria: that they were able to grow on amino-acid-free media and that the specimens appeared morphologically different from one another. Specimens from existing laboratory cultures were included to diversify our sample. We used amino-acid-free media to culture our microbial specimens to ensure that all the amino acids were synthesized de novo. The specimens were either cultured in liquid broth or on solid agar media (Bacto Agar BD, Sparks, Maryland, USA) with 15 g/L with

one of the three nutrient mixes here given with the carbon and nitrogen sources: Czapek (Sigma-Aldrich, St. Louis, Missouri, USA) with 30 g/L sucrose and 3 g/L sodium nitrate, modified arabinose gluconate (MAG; van Berkum 1990; modified from Cole and Elkan [1973]) with 1 g/L L-arabinose, 1 g/L D-gluconic acid, and 0.32 g/L ammonium chloride and Melin-Norkrans (MMN) agar medium (Marx 1969) with 10 g/L D-glucose and 0.25 g/L ammonium phosphate. The bulk δ^{13}C values of the substrates were ($N = 3$): bacto agar, $-19.6‰ ± 0.0‰$ (mean ± SE); D-glucose, $-9.5‰ ± 0.2‰$; Czapek, $-23.4‰ ± 0.0‰$; MAG with agar, $-20.0‰ ± 0.0‰$; and MMN with agar, $-14.4‰ ± 0.2‰$. Microbial cultures grown in liquid media were incubated at 25°C in 250-mL flasks in a shaking incubator (Innova 4230, New Brunswick Scientific, Edison, New Jersey, USA) and harvested after one to three weeks. Cultures grown on agar media were incubated at ambient temperature (22°C) in Petri dishes and harvested after one to five weeks. In addition to the laboratory-cultured fungi, we also collected fungal fruiting bodies in July 2007 at the boreal forest site to compare fungi with a variety of organic N sources to those cultured on amino-acid-free media.

Plants grown in the greenhouse were planted from seed in April 2007 and harvested in June 2007. They were grown on sphagnum and fertilized with Excel 15-5-15 Cal-Mag (Peters, Marysville, Ohio, USA). Plants from the boreal forest site were collected in July 2007. After collection, plant leaves were washed thoroughly in tap water to avoid contamination of soil and microorganisms.

Identification

The microbial specimens that we isolated or collected from the boreal forest site were taxonomically identified using rRNA gene sequence analyses (see Appendix A for methods). We identified all except three specimens at the level of genus or species based upon >96% sequence similarity to known entities (Table 1). The remaining three specimens, one fungus and two bacteria, were identified at the level of Division or Order. Nine out of 13 specimens belonged to the Phylum Ascomycota, three specimens to Basidiomycota (two of which were fruiting bodies collected in the boreal forest), and one specimen to Zygomycota. All three phyla are common in interior Alaska boreal forest soils, as described by Taylor et al. (2008). Of the 10 bacterial specimens in our study, seven specimens belonged to the Phylum Proteobacteria, two specimens to Actinobacteria, and one specimen to Bacteroidetes. This assemblage of phyla is representative of microbial diversity in Alaska soils, as found by Schloss and Handelsman (2006): 48.6% of bacterial 16S rRNA sequences belonged to Proteobacteria, 5.8% to Actinobacteria, and 9.3% to Bacteroidetes. Proteobacteria are gram-negative bacteria and Actinobacteria and Bacteroidetes are gram-positive.

TABLE 1. Species identification of organisms used in this study, their carbon sources, and their origins.

Identity	Phylogentic affiliation	Carbon source	Origin
Fungus			
Ascomycota	Ascomycota	Czapek liquid	isolated from boreal forest
Aureobasidium pullulans	Ascomycota	MAG liquid	isolated from boreal forest
Bionectria orhroleuc	Ascomycota	Czapek agar	isolated from boreal forest
Hypocrea pachybasioides	Ascomycota	Czapek agar	isolated from boreal forest
Lecythophora sp.	Ascomycota	Czapek agar	isolated from boreal forest
Nectria vilior	Ascomycota	Czapek liquid	isolated from boreal forest
Penicillium sp.	Ascomycota	Czapek liquid	isolated from boreal forest
Phoma glomerata	Ascomycota	Czapek agar	isolated from boreal forest
Trichoderma viridi	Ascomycota	Czapek agar	isolated from boreal forest
Cortinarius cystidi	Basidiomycota	Soil	harvested from boreal forest
Hericium racemosum	Basidiomycota	Czapek liquid	culture collection
Hydnum repandum	Basidiomycota	Soil	harvested from boreal forest
Mortierella alpina	Zygomycota	MMN agar	culture collection
Bacterium			
Actinobacteria	Actinobacteria	Czapek liquid	isolated from boreal forest
Rhodococcus spp.	Actinobacteria	Czapek liquid	culture collection
Bradyrhizobium sp.1	Alpha Proteobacteria	MAG agar	culture collection
Bradyrhizobium sp. 2	Alpha Proteobacteria	MAG agar	culture collection
Methylobacterium sp.	Alpha Proteobacteria	MAG liquid	isolated from boreal forest
Burkholderia xenovora	Beta Protobacteria	MAG liquid	culture collection
Escherichia coli	Gamma Proteobacteria	MAG liquid	culture collection
Gammaproteobacteria	Gamma Proteobacteria	Czapek agar	isolated from boreal forest
Klebsiella sp.	Gamma Proteobacteria	MAG agar	isolated from boreal forest
Pedobacter sp.	Sphingobacteria	MAG agar	isolated from boreal forest
Plant			
Calamagrostis purpurascens	Anthophyta	CO_2	harvested from boreal forest
Capsicum annuum	Anthophyta	CO_2	harvested from greenhouse
Carex bigelowii	Anthophyta	CO_2	harvested from boreal forest
Cucumis sativus	Anthophyta	CO_2	harvested from greenhouse
Helianthus annuus	Anthophyta	CO_2	harvested from greenhouse
Origanum vulgare	Anthophyta	CO_2	harvested from greenhouse
Solanum lycopersicum	Anthophyta	CO_2	harvested from greenhouse
Vaccinium uliginosum	Anthophyta	CO_2	harvested from boreal forest
Equisetum pratense	Pteridophyta	CO_2	harvested from boreal forest
Equisetum scirpoides	Pteridophyta	CO_2	harvested from boreal forest

Notes: All fungi and bacteria that were isolated from the boreal forest or originated from an existing culture collection were cultured and harvested in the laboratory. Three samples were discarded due to chromatographic co-elution or overloading: *Inocybe pudica* (wild fungus), *Hericium racemosum* (wild fungus), and *Bradyrhizobium* sp. 3 (culture collection bacterium). MAG is modified arabinose gluconate.

Amino acid extraction and isotope analysis

Samples were freeze dried and homogenized to a fine powder using a mortar and pestle and then weighed into 3–10 mg aliquots and transferred to Pyrex culture tubes (13 × 100 mm: Corning Glass, Corning, New York, USA). The samples were flushed with N_2 gas, sealed, and hydrolyzed in 1 mL HCl (6N Sequanal grade; Pierce Chemical, Rockford, Illinois, USA) at 110°C for 24 h. After hydrolysis, the samples were centrifuged at 600 g (1 gravity = 9.80665 m/s^2) for 3 min and the liquid phase transferred to 3.8 mL dram vials (VWR International, West Chester, Pennsylvania, USA) before evaporating the samples to dryness under a stream of N_2 gas for 30 min at 110°C. To volatize the amino acids, we derivatized the dried samples with acidified isopropyl and N-trifluoroacetate as described elsewhere (Silfer et al. 1991, O'Brien et al. 2002) with the exception that samples were evaporated to dryness at 60°C instead of at ambient temperature after the formation of amino acid isopropyl esters. To reduce oxidation during the derivatization, the vials were flushed or sealed with N_2 gas in every step involving heating of the samples. The

samples were derivatized in batches of six samples, including one mixed standard of 14 amino acids with known $\delta^{13}C$. After derivatization, the samples were stored in dichloromethane for two to three weeks at 4°C. Samples were prescreened with an Agilent 6890N gas chromatograph (GC; Agilent, Santa Clara, California, USA) to verify and optimize peak heights prior to isotope analysis.

Isotope measurements via GC-C-IRMS took place at the Alaska Stable Isotope Facility, UAF. Amino acid derivatives were injected into an Agilent 6890N gas chromatograph (GC) using an autosampler and separated on an HP Ultra-1 column (Agilent). The GC was interfaced with a Finnigan Delta Plus XP isotope ratio mass spectrometer (IRMS) via the GC-III combustion (C) interface (Thermo-Finnigan, Waltham, Massachusetts, USA). Data are expressed in delta notation as $((R_{smpl}/R_{stnd}) - 1) \times 1000‰$, where R is the ratio of heavy to light isotope, R_{smpl} is from the sample, and the standard (stnd) is Vienna PDB for carbon, as provided by pulses of calibrated CO_2 reference gas. We achieved consistently good chromatographic separation for 13

amino acids, here given in the ascending order of retention times: alanine (Ala), glycine (Gly), threonine (Thr), serine (Ser), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), asparagine (Asp), methionine (Met), glutamate (Glu), phenylalanine (Phe), and lysine (Lys) (see Appendix B). All samples were analyzed in triplicate. Amino acid $\delta^{13}C$ values were corrected for the carbon added during derivatization following O'Brien et al. (2002). Of 36 samples analyzed, three samples were discarded due to poor chromatography: *Inocybe pudica* (wild fungus), *Hericium racemosum* (wild fungus), and *Bradyrhizobium* sp. 3 (culture collection bacterium).

Statistical analysis

For the purposes of analysis we make an operational distinction between amino acids that have long biosynthetic pathways and are essential for animals and those with short biosynthetic pathways that are nonessential for animals, and analyze these data separately. We evaluated the factors affecting amino acid $\delta^{13}C$ values with a multivariate, nested ANOVA analysis, in which taxon (fungi, bacteria, or plant), growth medium, amino acid identity, and the taxon × amino acid interactions were the independent variables. Because some growth media were fungi- or bacteria-specific, growth medium was nested within taxon. We tested the normality of the residuals with Shapiro-Wilks test. Amino acid data used for graphical purposes and initial comparisons among taxa were normalized to the mean $\delta^{13}C$ ($\delta^{13}C_N$ = amino acid $\delta^{13}C$ − mean amino acid $\delta^{13}C$) to remove the affect of growth media. Essential amino acids and nonessential amino acids were normalized to their respective means. Differences between taxa for individual amino acids were found using one-way ANOVA with Tukey's hsd post hoc tests on amino acid $\delta^{13}C_N$ values. Data used in multivariate analysis were not normalized. We determined the set of $\delta^{13}C$ variations among all seven essential amino acids (the independent variables) that best classified the three taxa (the dependent variables) by applying linear discriminant analysis to maximize the ratio of between-taxon variance to within-taxon variance. We used Pillai-Bartlett trace to test whether the three taxa had different mean discriminant scores. All statistical analyses were performed in S-Plus version 6.2 (TIBCO, Palo Alto, California, USA). The linear discriminant function of the MASS library (Venables and Ripley 2002) was used for the linear discriminant analysis. Values are given as mean ± SE unless otherwise noted.

Validation

We tested our classification of fungal, bacterial, and plant amino acid synthesis based on the linear discriminant analysis of essential amino acid $\delta^{13}C$ values, using samples of plants (O'Brien et al. 2005), fungi (O'Brien et al. 2008), bacteria (Scott et al. 2006), and insect consumers with known plant or fungal diets (O'Brien et al. 2002, 2005, 2008). Most of these analyses were conducted in a different laboratory, but using nearly identical methods of derivatization and chromatography. The samples included 15 bacteria, three butterfly host plants, one yeast, five butterfly species, including at least two representatives of each, and six samples of fruit flies or fruit fly eggs grown on yeast (44 samples in total). For inclusion, the samples needed to have $\delta^{13}C$ measurements of Thr, Val, Leu, Ile, Phe, and Lys. Methionine was omitted from this list because it was not reported in any of these studies. We also attempted to classify three lichen specimens that we collected from the UAF boreal forest and one previously reported lichen (Scott et al. 2006). To classify these "unknown" samples into one of the three known taxa (fungi, bacteria, or plants) classified in this study we applied the prediction function of the linear discriminant analysis.

RESULTS

The identification of organisms used in this study is presented in Table 1. We measured $\delta^{13}C$ from 13 amino acids, seven essential (Thr, Val, Leu, Ile, Met, Phe, and Lys) and six nonessential (Ala, Gly, Ser, Pro, Asx, and Glx; see Appendix C). For both essential and nonessential amino acids, taxon, amino acid identity, and their interaction had highly significant effects on amino acid $\delta^{13}C$ (all $P < 0.0001$; see Appendix D). Growth medium also had a highly significant effect, as the different carbon sources varied significantly in $\delta^{13}C$ (see Appendix D). This ANOVA model accounted for most of the variation in our data set ($R^2 = 0.912$ for essential amino acids and 0.793 for nonessential amino acids). The presence of a highly significant interaction between taxon and amino acid demonstrates that isotopic variations among individual amino acids were taxon dependent. We examined these variations separately for essential and nonessential amino acids.

Essential amino acids

Fungi, bacteria, and plants exhibited very different patterns of $\delta^{13}C$ variation among essential amino acids (Fig. 1), especially between plants and bacteria. Variation in $\delta^{13}C$ values among the seven essential amino acids analyzed in this study was greatest in plants (ranging 20.9‰ ± 0.9‰), intermediate in fungi (ranging 11.8‰ ± 0.4‰), and smallest in bacteria (ranging 6.0‰ ± 1.1‰) (Fig. 1). Comparisons between taxa for each essential amino acid are presented in Table 2. Lysine and leucine exhibited highly significant differences in normalized ($\delta^{13}C_N$) values between all three taxa ($P < 0.0001$), as did threonine and valine ($P < 0.001$; Table 2). Isoleucine and methionine $\delta^{13}C_N$ values were significantly higher in fungi compared to plants and bacteria ($P < 0.0001$), but plants and bacteria were indistinguishable. Phenylalanine $\delta^{13}C_N$ values were the least variable among taxa; plants and fungi were significantly different ($P < 0.0001$), but the magnitude of that difference was quite small (2.3‰).
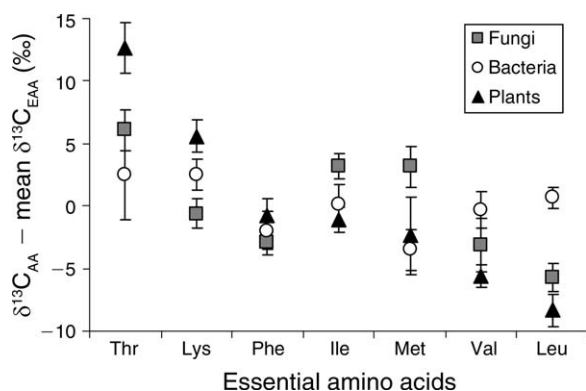
FIG. 1. The $\delta^{13}C$ values of individual essential amino acids (EAA) for each taxon, relative to their respective mean EAA $\delta^{13}C$. The error bars show $\pm SD$ of each taxon. The statistical significance of contrasts between the taxa is given in Table 1. Abbreviations are: Thr, threonine; Lys, lysine; Phe, phenylalanine; Ile, isoleucine; Met, methionine; Val, valine; Leu, leucine.

Linear discriminant analysis revealed highly significant differences between taxa, based on all non-normalized $\delta^{13}C$ values from seven essential amino acids (Pillai trace = 1.86, $F_{2,14} = 48.1$, $P < 0.0001$). The first discriminant axis separated fungi from plants and bacteria, and the second discriminant axis separated plants from fungi and bacteria, creating highly distinct isotopic clusters for each of the three taxa (Fig. 2A). All samples were classified with >99.99% certainty. The essential amino acids that correlated the most strongly with the two linear discriminant axes were Leu, Ile, and Lys (all coefficients > 0.5; Appendix C).

In our sample set we included wild specimens of plants and fungi, which have access to soil amino acids (Näsholm et al. 1998). To test whether wild and cultured plants clustered differently due to the influence of exogenous amino acids or other factors, we classified them separately before conducting linear discriminant analysis. We did not find any evidence that wild and cultured specimens had different ^{13}C fingerprints, as their clusters overlapped in the linear discriminant plot. We did not have sufficient sample sizes of wild-collected fungi to test wild and cultured separately, but all fungal samples made a distinct cluster regardless of origin (Fig. 2A). For this reason we subsequently did not draw a distinction between wild and cultured specimens in our analyses.

We also examined whether samples could be classified using $\delta^{13}C$ values from fewer amino acids. For this purpose we selected the three most informative amino acids: Leu, Ile, and Lys. The linear discriminant model with only three amino acids was highly significant (Pillai trace = 1.77, $F_{2,6} = 76.1$, $P < 0.0001$), separating bacteria from plants on the first linear discriminant axis and fungi from bacteria and plants on the second linear discriminant axis (see Appendix E). All samples were classified with >99.99% certainty. However, the overall separation was stronger using seven amino acids.

Simply plotting the differences in $\delta^{13}C$ values between the three most informative amino acids (Ile–Leu vs. Ile–Lys) created three highly distinct clusters for each taxon (Fig. 3). Thus, multivariate statistics, while valuable for making statistical assignments, are not necessary to visualize the isotopic clustering of each group.

Nonessential amino acids

Our ANOVA analysis of nonessential amino acids also revealed significant interactions between taxa and amino acid identities (all $P < 0.0001$; see Appendix C). The pattern of amino acid variation in $\delta^{13}C_N$ values was very similar between fungi and bacteria and different from that of plants (see Appendix F). When we compared $\delta^{13}C_N$ values between fungi, bacteria, and plants for each nonessential amino acid, fungi and bacteria were never different (Table 2). Bacteria and plants only differed in Ala and Asx, and plants differed significantly from fungi in all nonessential amino acid except Ser (Table 2). Our linear discriminant model for the nonessential amino acids was significant (Pillai trace = 1.11, $F_{2,12} = 5.50$, $P < 0.001$) but with a poorer separation than with essential amino acids; the clusters for bacteria and fungi overlapped and the clusters for fungi and plants were very close (Fig. 2B).

Classification/validation

Our linear discriminant model correctly classified 40 out of 46 samples (87%) from previously published studies (Table 3). Essential amino acids from the host plants and the butterflies that fed on them as larvae were classified as plants (probability > 96%), with the exception of two butterflies (*Heliconius charitonia*) that

TABLE 2. Significant differences between normalized $\delta^{13}C$ amino acid values from fungi ($N = 13$), bacteria ($N = 10$), and plants ($N = 10$).

Comparison	Essential amino acids (EAA)							Nonessential amino acids (NAA)					
	Ile	Leu	Lys	Met	Phe	Thr	Val	Ala	Asp	Glu	Gly	Pro	Ser
Bacteria–fungi	§	§	§	§		†	†						
Bacteria–plants		§	§			§	§	§	‡				
Fungi–plants	§	§	§	§	‡	§	†	§	†	†	§	‡	

Notes: Abbreviations are: Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Thr, threonine; Val, valine; Ala, alanine; Asp, asparagine; Glu, glutamate; Gly, glycine; Pro, proline; Ser, serine. Confidence intervals: † ≥99%, ‡ ≥99.9%, § ≥99.99%.
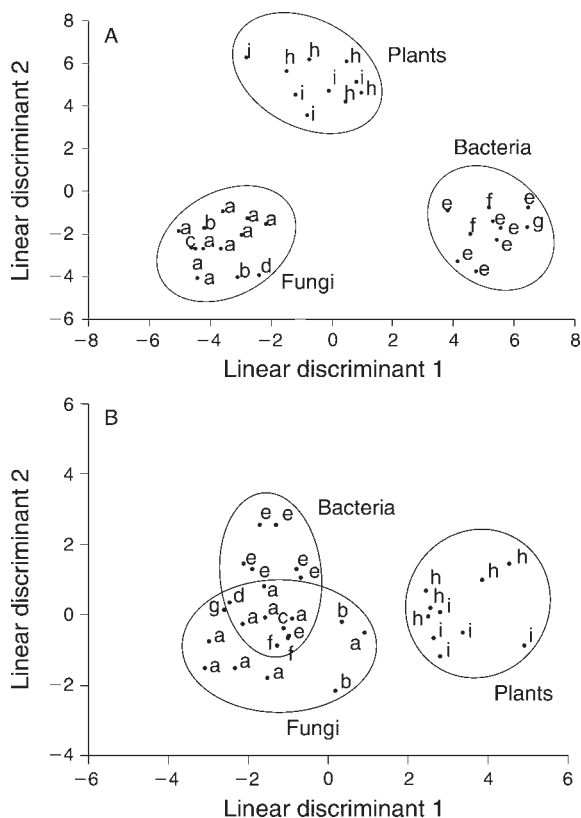
FIG. 2. Linear discriminant analysis of fungi ($N = 13$), bacteria ($N = 10$), and plants ($N = 10$) using ^{13}C variations among (A) the seven essential amino acids (threonine, valine, leucine, isoleucine, methionine, phenylalanine, and lysine) and (B) the six nonessential amino acids (serine, asparagine, glycine, proline, glutamate, and alanine). Letters signify: a, Ascomycota culture; b, Basidomycota collected: c, Basidomycota culture; d, Zygomycota culture; e, proteobacteria culture; f, actinobacteria culture; g, Bacteroidetes culture; h, greenhouse plants; i, wild plants.

were categorized as bacteria (probability = 77%) primarily due to their Lys and Val $\delta^{13}C$ values. Essential amino acids from both the yeast and the fruit flies that fed on them were correctly classified as fungal in origin. Of the fifteen bacterial samples reported in Scott et al. (2006), 10 were categorized as bacteria (probability = 100%), three could not be reliably classified, and two were miscategorized as plants (Table 3). All four lichen samples, three from this study and one from Scott et al. (2006), were classified as fungi with a very high probability (probability = 100%; Table 3).

DISCUSSION

This study demonstrates for the first time that fungi, bacteria, and plants generate distinct patterns of essential amino acid ^{13}C during biosynthesis and that these patterns or fingerprints can be used to identify the origin of amino acids even when there is considerable variability in bulk $\delta^{13}C$. The C_3 plants were characterized by enriched Thr and bacteria by enriched Leu,

consistent with three recent studies examining amino acid $\delta^{13}C$ in five plant species and 21 bacterial species (O'Brien et al. 2003, 2005, Scott et al. 2006). We found the isotopic difference between Ile and Leu to be particularly valuable in distinguishing plant and fungal from bacterial samples (Fig. 3), in that the difference is on the order of 6–12‰ in plants and fungi and −3 to 2‰ in bacteria. This result is consistent with all the previous studies we examined, dating back to the early 1960s (Abelson and Hoering 1961, Winters 1971, Macko et al. 1987, Hare et al. 1991, Fogel and Tuross 1999, Abraham and Hesse 2003, O'Brien et al. 2003, 2005, Scott et al. 2006) with the exception of a single fungal specimen (Abraham and Hesse 2003) and a bacterium grown on glutamate medium (Macko et al. 1987). The contrast of Thr and Leu was informative, but more variable and less consistent with previous studies. Fungi exhibited enriched Ile and Met, and the contrast between Ile and Lys was particularly useful for separating fungal from plant samples (Figs. 1 and 3). While discriminant function analysis provides the most robust way to classify samples, we found that samples could be categorized based only on the three most informative amino acids: Ile, Leu, and Lys (Fig. 3; Appendix E).

When we applied our linear discriminant function to previously published data from plants (three; O'Brien et al. 2005), fungi (one; O'Brien et al. 2008), and bacteria (15; Scott et al. 2006), as well as insect consumers feeding on plants (18; O'Brien et al. 2002, 2005) and fungi (six; O'Brien et al. 2008), the model classified all plants, all fungi, most (67%) of the bacteria, and most (92%) of the consumers correctly (based on diet). The model's success in assigning consumers was particularly
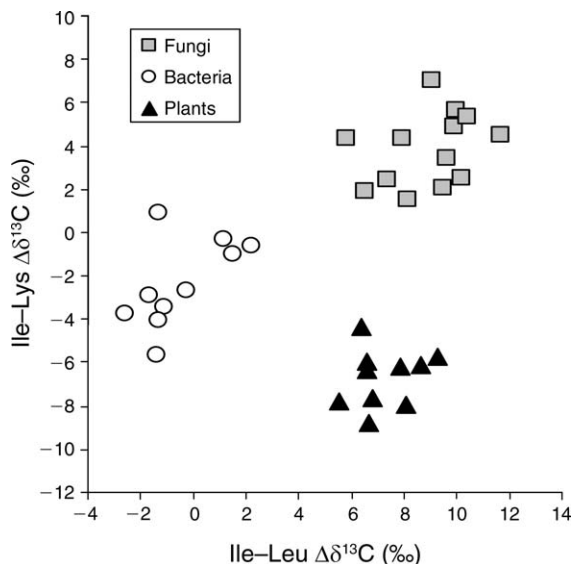

FIG. 3. Differences in amino acid ^{13}C values between the three most informative essential amino acids (Ile, isoleucine; Lys, lysine; and Leu, leucine) result in three distinct clusters separating fungi, bacteria, and plants.

TABLE 3. Predicting classification of amino acid ^{13}C samples of our own and previous studies based on the linear discriminant analysis of the 33 fungi (F), bacteria (B), and plant (P) samples of this study.

Sample	N	Actual	Pre-dicted	Probability (%)
Plants				
Vicia villosa[1]	1	P	P	100
Viola soraria[1]	1	P	P	100
Passiflora caerulea[1]	1	P	P	100
Fungi				
Saccharomyces cerevisiae[2]	3	F	F	100
Lichens				
Cetraria pinastri	1	U	F	100
Parmelia sulcata	1	U	F	100
Peltigera apthosa	1	U	F	100
Antarctic lichen[3]	1	U	F	100
Bacteria				
Escherichia coli[3]				
Aerobic, pyruvate	1	B	B	100
Anaerobic, pyruvate	1	B	B	100
Acetate	1	B	B	100
Shewanella oneidensis[3]				
Aerobe, fumarate	1	B	B	100
Aerobe, nitrate	1	B	B	100
Anaerobe, fumarate	1	B	F	64
Anaerobe, nitrate	1	B	P	51
Desulfovibrio[3]	1	B	P	100
Methylophilus[3]	1	B	B	100
Methylobacterium[3]				
Methylamine	1	B	B	100
Methanol	1	B	B	100
Clostridium acetobutylicum[3]	1	B	P	99
Desulfotomaculum[3]	1	B	B	100
Aquifex[3]	1	B	B	100
Thermotoga[3]	1	B	F	74
Consumers				
Colias villosa eggs[1]	4	P	P	98
Speyeria mormonia eggs[1]	4	P	P	96
Euphydryas chalcedona eggs[1]	4	P	P	100
Heliconius charitonia eggs[1]	2	P	B	77
Amphion floridensis eggs[4]	4	P	P	100
Drosophila melanogaster adults and eggs[2]	6	F	F	100

Notes: Consumers were expected to have the same classification as their diets because only essential amino acids (threonine, valine, leucine, isoleucine, phenylalanine, and lysine) were included in this analysis. The actual classification of the three lichen species was unknown (U) because they are symbionts between cyanobacteria and fungi. The lichens *Cetraria pinastri*, *Parmelia sulcata*, and *Peltigera apthosa* were collected from the boreal forest site May 2007 and analyzed along with the other samples of this study. Sources are identified by superscripts: 1, O'Brien et al. (2005); 2, O'Brien et al. (2008); 3, Scott et al. (2006); 4, O'Brien et al. (2002).

encouraging, as one of the most obvious applications for an amino acid ^{13}C fingerprint would be to identify dietary sources of essential amino acids in animals with unknown or mixed diets. That the performance of the model was not quite as good for bacterial samples is perhaps not surprising given the variety of growing conditions and great diversity of bacterial taxa in the study by Scott et al. (2006); certainly the diversity

exceeded that used to "train" the linear discriminant model in the present study. If we generated a new discriminant analysis including Scott's bacterial samples as well as those of bacteria, plants, and fungi from the present study, only one specimen, *Desulfuvibrio*, fell outside this more broadly defined bacterial fingerprint (see Appendix G). This data set loses some discriminatory power by omitting Met but demonstrates the distinctness of the bacterial fingerprint over a broader range of taxa. The classification of the four lichens as fungi illustrates how ^{13}C fingerprints can be used to investigate amino acid synthesis by symbiotic relationships, with the caveat that we did not sample the photobionts, green algae and cyanobacteria. We conclude that the method is fairly robust even when applied across different laboratories and studies. However, because analytical conditions can vary, we want to emphasize that the best way to classify dietary sources based on amino acids would be to analyze known potential amino acid sources alongside the samples of interest.

Lysine was among the most informative amino acids, exhibiting little within-taxa variation and distinct clusters for each group. However, the enrichment of Lys in bacteria relative to plants is not consistent with data compiled from recent previous studies (O'Brien et al. 2002, 2005, Scott et al. 2006). In plants and bacteria, Lys biosynthesis is known to occur via a pathway that utilizes aspartate as a precursor and diaminopimelic acid (DAP) as an intermediate (Hudson et al. 2005, 2006). Four established variants of that pathway are known, of which one is specific to plants and bypasses three reactions otherwise found in bacteria. In contrast, fungi synthesize Lys by the α-aminoadipate pathway, which uses 2-oxoglutarate as a precursor (Nishida and Nishiyama 2000). Thus, plants, fungi, and prokaryotes use different precursors, intermediates, and reactions in Lys biosynthesis, and these differences generate a distinct Lys signature in each taxon, relative to average amino acid $\delta^{13}C$. The lack of consistency of bacterial Lys between this study and Scott et al. (2006) may be an effect of taxonomic sampling, given that at least three distinct Lys biosynthesis pathways exist in bacteria (Kosuge and Hoshino 1999, Vauterin et al. 2000).

Members of the pyruvate family (Ala, Val, Leu, and Ile) were also highly informative in distinguishing the biosynthetic origin of amino acids, particularly in distinguishing plants from fungi and bacteria. In contrast to Lys biosynthesis, the pathways leading to synthesis of the amino acids of the pyruvate family are similar in fungi, bacteria, and plants (Hagelstein et al. 1997, Kohlhaw 2003, Keseler et al. 2005). However, several studies have demonstrated that organisms have different isotope effects associated with pyruvate dehydrogenase, which could cause differential enrichment of the pool of available pyruvate for biosynthesis (Blair et al. 1985, Melzer and Schmidt 1987). Our data suggest in particular that pyruvate may be especially ^{13}C depleted

in plants, as Ala, which is synthesized in one step from pyruvate, was depleted in plants by 4–5‰ relative to fungi and bacteria. Plants also exhibited a larger depletion of Ile relative to Thr compared to fungi and bacteria, which reflects the addition of acetyl carbon from pyruvate.

We found that plants, fungi, and bacteria generated distinctly different isotope fingerprints in spite of organisms from each lineage being grown on a range of different carbon sources, from soil to amino-acid-free broth. This is promising for moving the isotope fingerprinting tool to the field, since the $\delta^{13}C$ values of individual amino acids within one lineage ranged more than 20‰, depending on the particular growth medium. However, it needs to be addressed in detail how external conditions and amino acid availability may affect isotope variation. Because microorganisms have enormous flexibility within central metabolism to supply precursors for the synthesis of amino acids (Walsh and Koshland 1985, Marx et al. 1997), further studies on how growing conditions induce isotope variations would be useful for applying isotope fingerprinting to field studies. Likewise, it needs to be systematically addressed how external amino acids are routed or processed in organisms such as boreal plants, for which amino acids are considered to be an important nitrogen source (Näsholm et al. 1998, Chalot et al. 2006, Kielland et al. 2006). For the limited number of samples in our study, we did not find any difference in patterns of amino acid $\delta^{13}C$ between plants collected in the boreal forest and plants cultured in the greenhouse. Finally, to provide a wider scope for applying the isotope fingerprinting tool in ecological studies it would be useful to expand the ^{13}C fingerprinting library of C_3 plants to include C_4 and crassulacean acid metabolism (CAM) plants, since their different modes of carbon acquisitions and associated metabolic fluxes could yield different isotope fingerprints.

Classification models such as linear discriminant analysis are suitable for predicting membership in one or another group, but do not effectively estimate the relative contributions of two or more sources to a mixed sample, as would a mixing model (Phillips and Gregg 2003). We found that plotting the differences in $\delta^{13}C$ values between the three most informative amino acids (Ile–Leu vs. Ile–Lys) created three distinct clusters for each taxon (Fig. 3). We propose that such differences could serve as the endpoints in a dietary mixing model (Phillips 2001, Phillips and Gregg 2003) to demonstrate the relative input of plant, fungal, and microbial essential amino acids to an animal's diet. We only included three amino acids for the purpose of visualization, but mixing models can include a large number of endpoints as long as there are sufficient isotopic dimensions to resolve them. Thus, contrasts between essential amino acid $\delta^{13}C$ could provide a tool for estimating the contribution of diet sources to consumers.

In conclusion, we found that fungi, bacteria, and plants impart characteristic patterns of ^{13}C variation during synthesis of amino acids, particularly those with longer and more complex pathways that are essential in animal diets. Because these patterns are diagnostic of the biosynthetic origin of amino acids, they can be used as a ^{13}C fingerprint to assign unknown samples or sources of amino acids as plant, fungal, or bacterial. Lysine, Leu, and Ile were, in combination, the most informative amino acids; however, samples were categorized with the greatest accuracy using all of the essential amino acids measured. Our results suggest that ^{13}C fingerprinting of amino acids could provide a powerful in situ assay of amino acid sources in terrestrial ecosystems, whether for symbiotic associations between animals and microorganisms and between plants and fungi or in identifying the primary contributors of amino acids in animals.

LITERATURE CITED

Abelson, P. H., and T. C. Hoering. 1961. Carbon isotope fractionation in formation of amino acids by photosynthetic organisms. Proceedings of the National Academy of Sciences (USA) 47:623–632.

Abraham, W. R., and C. Hesse. 2003. Isotope fractionations in the biosynthesis of cell components by different fungi: a basis for environmental carbon flux studies. Fems Microbiology Ecology 46:121–128.

Baumann, P., N. A. Moran, and L. Baumann. 2006. Bacteriocyte-associated endosymbionts of insects. Pages 403–438 in M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer, and E. Stackebrandt, editors. The Prokaryotes. Springer, New York, New York, USA.

Blair, N., A. Leu, E. Munoz, J. Olsen, E. Kwong, and D. Desmarais. 1985. Carbon isotopic fractionation in heterotrophic microbial-metabolism. Applied and Environmental Microbiology 50:996–1001.

Brundrett, M. 2004. Diversity and classification of mycorrhizal associations. Biological Reviews 79:473–495.

Chahartaghi, M., R. Langel, S. Scheu, and L. Ruess. 2005. Feeding guilds in Collembola based on nitrogen stable isotope ratios. Soil Biology and Biochemistry 37:1718–1725.

Chalot, M., D. Blaudez, and A. Brun. 2006. Ammonia: a candidate for nitrogen transfer at the mycorrhizal interface. Trends in Plant Science 11:263–266.

Cole, M. A., and G. H. Elkan. 1973. Transmissible resistance to penicillin-G, neomycin, and chloramphenicol in Rhizobium japonicum. Antimicrobial Agents and Chemotherapy 4:248–253.

Fantle, M. S., A. I. Dittel, S. M. Schwalm, C. E. Epifanio, and M. L. Fogel. 1999. A food web analysis of the juvenile blue

crab *Callinectes sapidus* using stable isotopes in whole animals and individual amino acids. Oecologia 120:416–426.

Fogel, M. L., and N. Tuross. 1999. Transformation of plant biochemicals to geological macromolecules during early diagenesis. Oecologia 120:336–346.

Gietzen, D. W., S. Hao, and T. G. Anthony. 2007. Mechanisms of food intake repression in indispensable amino acid deficiency. Annual Review of Nutrition 27:63–78.

Hagelstein, P., B. Sieve, M. Klein, H. Jans, and G. Schultz. 1997. Leucine synthesis in chloroplasts: leucine/isoleucine aminotransferase and valine aminotransferase are different enzymes in spinach chloroplasts. Journal of Plant Physiology 150:23–30.

Hare, P. E., M. L. Fogel, T. W. Stafford, A. D. Mitchell, and T. C. Hoering. 1991. The isotopic composition of carbon and nitrogen in individual amino acids isolated from modern and fossil proteins. Journal of Archaeological Science 18:277–292.

Hayes, J. M. 1993. Factors controlling ^{13}C contents of sedimentary organic compounds: principles and evidence. Marine Geology 113:111–125.

Hayes, J. M. 2001. Fractionation of the isotopes of carbon and hydrogen isotopes in biosynthetic processes. Pages 225–277 *in* J. W. Valley and D. R. Cole, editors. Reviews in mineralogy and geochemistry 43. Stable isotope geochemistry. Mineralogical Society of America, Washington, D.C., USA.

Hudson, A. O., C. Bless, P. Macedo, S. P. Chatterjee, B. K. Singh, C. Gilvarg, and T. Leustek. 2005. Biosynthesis of lysine in plants: evidence for a variant of the known bacterial pathways. Biochimica et Biophysica Acta, General Subjects 1721:27–36.

Hudson, A. O., B. K. Singh, T. Leustek, and C. Gilvarg. 2006. An LL-diaminopimelate aminotransferase defines a novel variant of the lysine biosynthesis pathway in plants. Plant Physiology 140:292–301.

Janson, E. M., J. O. Stireman, M. S. Singer, and P. Abbot. 2008. Phytophagous insect–microbe mutualisms and adaptive evolutionary diversification. Evolution 62:997–1012.

Keseler, I. M., J. Collado-Vides, S. Gama-Castro, J. Ingraham, S. Paley, I. T. Paulsen, M. Peralta-Gil, and P. D. Karp. 2005. EcoCyc: a comprehensive database resource for *Escherichia coli*. Nucleic Acids Research 33:D334–D337.

Kielland, K., J. McFarland, and K. Olson. 2006. Amino acid uptake in deciduous and coniferous taiga ecosystems. Plant and Soil 288:297–307.

Kohlhaw, G. B. 2003. Leucine biosynthesis in fungi: entering metabolism through the back door. Microbiology and Molecular Biology Reviews 67:1–15.

Kosuge, T., and T. Hoshino. 1999. The alpha-aminoadipate pathway for lysine biosynthesis is widely distributed among Thermus strains. Journal of Bioscience and Bioengineering 88:672–675.

Larsen, T., A. Gorissen, P. H. Krogh, M. Ventura, and J. Magid. 2007. Assimilation dynamics of soil carbon and nitrogen by wheat roots and Collembola. Plant and Soil 295:253–264.

Lehninger, A. L. 1975. Biochemistry. Worth, New York, New York, USA.

Macko, S. A., M. L. Fogel, P. E. Hare, and T. C. Hoering. 1987. Isotopic fractionation of nitrogen and carbon in the synthesis of amino-acids by microorganisms. Chemical Geology 65:79–92.

Marx, A., K. Striegel, A. A. deGraaf, H. Sahm, and L. Eggeling. 1997. Response of the central metabolism of *Corynebacterium glutamicum* to different flux burdens. Biotechnology and Bioengineering 56:168–180.

Marx, D. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163.

McCarthy, M. D., R. Benner, C. Lee, J. I. Hedges, and M. L. Fogel. 2004. Amino acid carbon isotopic fractionation patterns in oceanic dissolved organic matter: An unaltered photoautotrophic source for dissolved organic nitrogen in the ocean? Marine Chemistry 92:123–134.

Meier-Augenstein, W. 1999. Applied gas chromatography coupled to isotope ratio mass spectrometry. Journal of Chromatography A 842:351–371.

Melzer, E., and H. L. Schmidt. 1987. Carbon isotope effects on the pyruvate–dehydrogenase reaction and their importance for relative ^{13}C depletion in lipids. Journal of Biological Chemistry 262:8159–8164.

Moran, N. A., and P. Baumann. 2000. Bacterial endosymbionts in animals. Current Opinion in Microbiology 3:270–275.

Näsholm, T., A. Ekblad, A. Nordin, R. Giesler, M. Högberg, and P. Högberg. 1998. Boreal forest plants take up organic nitrogen. Nature 392:914–916.

Nishida, H., and M. Nishiyama. 2000. What is characteristic of fungal lysine synthesis through the α-aminoadipate pathway? Journal of Molecular Evolution 51:299–302.

O'Brien, D. M., C. L. Boggs, and M. L. Fogel. 2003. Pollen feeding in the butterfly *Heliconius charitonia*: isotopic evidence for essential amino acid transfer from pollen to eggs. Proceedings of the Royal Society B 270:2631–2636.

O'Brien, D. M., C. L. Boggs, and M. L. Fogel. 2005. The amino acids used in reproduction by butterflies: a comparative study of dietary sources using compound-specific stable isotope analysis. Physiological and Biochemical Zoology 78:819–827.

O'Brien, D. M., M. L. Fogel, and C. L. Boggs. 2002. Renewable and nonrenewable resources: amino acid turnover and allocation to reproduction in Lepidoptera. Proceedings of the National Academy of Sciences (USA) 99:4413–4418.

O'Brien, D. M., K.-J. Min, T. Larsen, and M. Tatar. 2008. Use of stable isotopes to examine how dietary restriction extends Drosophila lifespan. Current Biology 18:R155–R156.

Phillips, D. L. 2001. Mixing models in analyses of diet using multiple stable isotopes: a critique. Oecologia 127:166–170.

Phillips, D. L., and J. W. Gregg. 2003. Source partitioning using stable isotopes: coping with too many sources. Oecologia 136:261–269.

Read, D. J., and J. Perez-Moreno. 2003. Mycorrhizas and nutrient cycling in ecosystems—a journey towards relevance? New Phytologist 157:475–492.

Robbins, C. T. 1993. Wildlife feeding and nutrition. Second edition. Academic Press, San Diego, California, USA.

Schloss, P. D., and J. Handelsman. 2006. Toward a census of bacteria in soil. PLoS Computational Biology 2:786–793.

Scott, J. H., D. M. O'Brien, D. Emerson, H. Sun, G. D. McDonald, A. Salgado, and M. L. Fogel. 2006. An examination of the carbon isotope effects associated with amino acid biosynthesis. Astrobiology 6:867–880.

Silfer, J. A., M. H. Engel, S. A. Macko, and E. J. Jumeau. 1991. Stable carbon isotope analysis of amino acid enantiomers by conventional isotope ratio mass spectrometry and combined gas chromatography/isotope ratio mass spectrometry. Analytical Chemistry 63:370–374.

Stephanopoulos, G. N., A. A. Aristidou, and J. Nielsen. 1998. Metabolic engineering: principles and methodologies. Academic Press, San Diego, California, USA.

Taylor, D. L., M. G. Booth, J. W. Mcfarland, I. C. Herriott, N. J. Lennon, C. Nusbaum, and T. G. Marr. 2008. Increasing ecological inference from high throughput sequencing of fungi in the environment through a tagging approach. Molecular Ecology Resources 8:742–752.

Van Berkum, P. 1990. Evidence for a third uptake hydrogenase phenotype among the soybean bradyrhizobia. Applied and Environmental Microbiology 56:3835–3841.

Vauterin, M., V. Frankard, and M. Jacobs. 2000. Functional rescue of a bacterial dapA auxotroph with a plant cDNA

library selects for mutant clones encoding a feedback-insensitive dihydrodipicolinate synthase. Plant Journal 21:239–248.

Venables, W. N., and B. D. Ripley. 2002. Modern applied statistics with S-Plus. Fourth edition. Springer-Verlag, New York, New York, USA.

Walsh, K., and D. E. Koshland. 1985. Branch point control by the phosphorylation state of isocitrate dehydrogenase: a quantitative examination of fluxes during a regulatory transition. Journal of Biological Chemistry 260:8430–8437.

Wernegreen, J. J. 2002. Genome evolution in bacterial endosymbionts of insects. Nature Reviews: Genetics 3:850–861.

Winters, J. K. 1971. Variations in the natural abundance of ^{13}C in proteins and amino acids. Dissertation. University of Texas, Austin, Texas, USA.

APPENDIX A

Method description of rRNA gene sequence analyses of fungi and bacteria (*Ecological Archives* XX-XXX-XXXX).

APPENDIX B

The $\delta^{13}C$ values of amino acids from fungi, bacteria, and plants (*Ecological Archives* XX-XXX-XXXX).

APPENDIX C

Summary of linear discriminant analysis of ^{13}C variation among seven and three essential amino acids (*Ecological Archives* XX-XXX-XXXX).

APPENDIX D

Results of ANOVA with factors affecting amino acid $\delta^{13}C$ values (*Ecological Archives* XX-XXX-XXXX).

APPENDIX E

Linear discriminant analysis of fungi, bacteria, and plants using ^{13}C variations among the three most informative amino acids (*Ecological Archives* XX-XXX-XXXX).

APPENDIX F

The $\delta^{13}C$ values of individual nonessential amino acids for each taxon relative to their respective means (*Ecological Archives* XX-XXX-XXXX).

APPENDIX G

Linear discriminant analysis using ^{13}C variations among six essential amino acids of the present study and those of Scott et al. (2006) (*Ecological Archives* XX-XXX-XXXX).