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Combined Genomic−Metabolomic Approach for the Differentiation of Geographical Origins of Natural Products: Deer Antlers As an Example

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Supporting Information

ABSTRACT: The correct identification of the geographical origin of deer antlers is essential to quality control, as its positive physiological effects correlate with chemical components. In this study, we applied both genomics and metabolomics to the origin-identification of 101 samples from Canada, New Zealand, and Korea. The genomics identified deer species in each country but failed to categorize all the samples, due to the presence of identical species in different countries. For identical species, NMR-based metabolomics gave clean separations, compounds specific to each country were identified, and the validity was confirmed by prediction analysis. As the genomics provided unambiguous read-outs for different species, and the metabolomics cleanly distinguished among identical species from different countries, their combined use could be a robust method for origin-identification even in difficult cases. We believe the method to be generally applicable to many herbal medicinal products for which various species are grown internationally.

KEYWORDS: Deer antler, *Cervus elaphus*, genomics, metabolomics, NMR

INTRODUCTION

In East Asian countries, deer antlers have been used as important medicinal ingredients and dietary supplements for hundreds of years. In fact, much research has shown that deer antlers can provide beneficial antiaging[1] and anti-inflammatory,[2] as well as blood-regenerating and blood-pressure-lowering effects.[3] These salutary effects are due to the chemicals and other constituents that have been found to be present in deer antlers: amino acids, nucleic acids, polyamines, vitamins, and additional organic and inorganic acids.[4] As with any other natural products, deer antler compositions vary quite widely according to geographical origins, and prices also vary, as much as 10-fold. Almost inevitably then, fraud and illicit trade have ensued, causing socioeconomic problems in antler-producing countries and medical malpractice issues in antler-consuming countries. As various species of deer are cultivated in different countries, classification of the origins of deer antlers is essential for their legal marketing and correct use.

Studies have differentiated deer or antlers using genetic, morphological, and metabolomic approaches.[5−11] A phylogenetic analysis of the antler-growing genus *Cervus* was performed using the entire cytochrome *b* gene,[12] and a world deer phylogeny was constructed on the basis of mitochondrial DNA (mtDNA) gene sequence data.[13,14] Classification of deer species according to their genetic variation has been conducted by karyotyping,[5] repetitive DNA sequencing,[6,7] RFLP analysis of mtDNA,[8] and gene sequencing of mtDNA.[15] Deer have been classified also on a morphological basis: European red deer (*C. elaphus*), wapiti (the *C. elaphus* subspecies of Asia and North America), and sika deer (*Cervus nippon*).[12] They are very similar to one another, except in body size and antler morphology.[9,10,12] Last, a metabolomic approach combined with principal component analysis (PCA) has been employed for the classification of antlers.[11] However, the number of samples in one group was quite small (about six), and the statistical approach cannot be used for the prediction of unknown samples.

Although the above approaches have their respective merits, drawbacks limit their general use: genetic approaches may not be practical for differentiating antlers from the same species; morphological studies lack objective criteria; and metabolomic approaches require sizable sample sizes for their statistical reliability. In the present study, we employed a combined genomic−metabolomic approach to determine various species' origins. We used an approximately five-times-larger sample size, for one country group, than did the previous study.[11] Whereas neither individual approach, genomic or metabolomic, proved fully satisfactory, their combination enabled accurate and reliable differentiation of deer antler origin from among the various species. Origin prediction proved effective even for the same genetic species from different countries. Our method can be applied to the discrimination not only of deer antlers but also, more generally, of other Oriental medicines such as herbs and plants.

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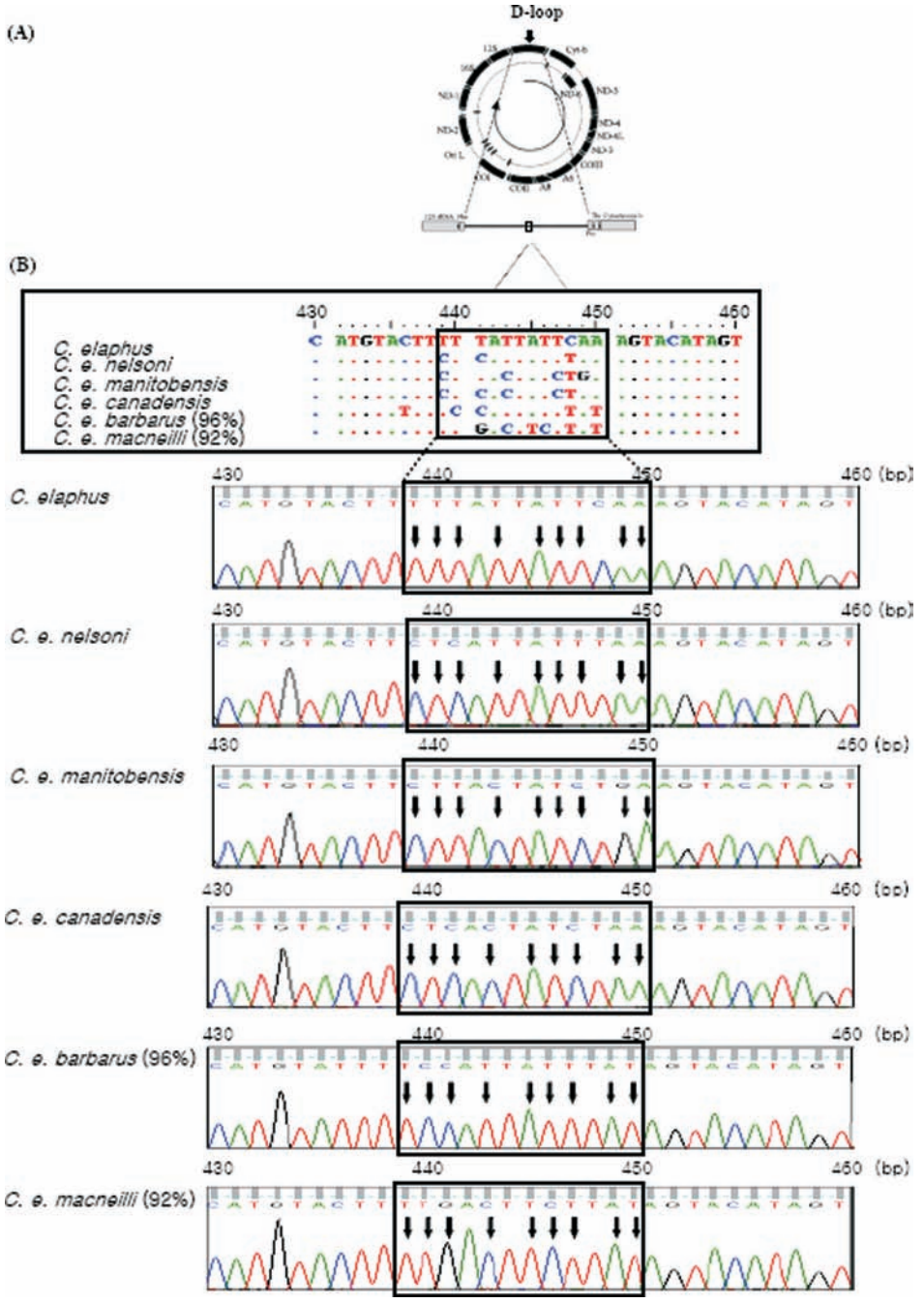

Figure 1. DNA sequence analysis of deer antler samples. The sequence amplified from the D-loop region of mtDNA using CST2 and CST39 primers were compared with those available in the NCBI database. (A) Schematic illustration of the mitochondrial DNA. (B) Representative chromatograms of the DNA sequencing results are shown for all identified species. Black arrows indicate where the sequences vary according to the species.

MATERIALS AND METHODS

Sample Collection. All deer antler samples were collected from deer bred on local farms that have research contracts with Korea Nokyong Research Center in Konkuk University (Chungju, Korea). The samples were collected in person by us during visits to the farms in the respective countries. A total of 101 deer antler samples were collected from several origins in Canada (40 samples), New Zealand (30 samples), and Korea (31 samples). The antlers were obtained from deer after 80 days of growth of their new horns, ground with a mortar and pestle, and stored in plastic bags at −80 °C until use.

DNA Sequence Analysis. The species of all the samples were determined by analyzing the nucleotide sequence of the D-loop region of mitochondrial DNA (Figure 1A). The sequences were compared with those available in the NCBI database by means of BLAST searches which allowed us to find regions of local similarity among sequences.

DNA was extracted from tissue using the QIAamp DNA microkit (QIAGEN, Duesseldorf, Germany) according to the protocol described by the manufacturer. To amplify the D-loop region of mtDNA from extracted total genomic DNA, CST2 (5′-taatatactggtcttgtaaacc-3′) and CST39 (5′-gggtcggaaggctgggaccaaacc-3′) primers were used. All reactions were carried out in a total final volume of 25 μL using 1 ng of DNA

as a template. The reaction mixtures contained 10× reaction buffer (Applied Biosystems, California, USA), 1.5 mM $MgCl_2$, and 0.2 mM dNTP mixture, along with 1.25 units of AmpliTaq Gold polymerase (Applied Biosystems, California, USA). Amplification was conducted in a GeneAmp PCR System 9700 (Applied Biosystems, California, USA) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension step at 72 °C for 7 min. The PCR products were sequenced in order to assess their identity. After determining the nucleotide sequence with ABI 3730 (Applied Biosystems, California, USA), it was analyzed by using DNASIS MAX, version 2.05 (MiraiBio, San Francisco, USA).

Sample Preparation for NMR Spectroscopy. One hundred milligrams of ground deer antlers was measured and put into 2 mL centrifuge tubes. The samples were extracted with buffer composed of a mixture of CD_3OD and D_2O (v/v, 1:1), 10 mM sodium phosphate (pH 6.0), and 0.025% (w/v) TMSP (trimethylsilylpropionic acid sodium salt-d_4) as an internal standard. The extraction was carried out by sonication at room temperature for 20 min. After centrifugation (13,000*g* for 10 min), the supernatant was transferred to a 5 mm NMR tube.[16]

NMR Spectroscopy. One-dimensional NMR spectra were measured on a 500-MHz Bruker Avance spectrometer equipped with a cryogenic triple resonance probe (Bruker, Billerica, MA, USA). The parameters were the same as those previously reported.[17] The time domain data were Fourier-transformed, phase-corrected, and baseline-corrected manually. The signal intensities were normalized to the intensity of the internal standard (0.025% TMSP) at 0.00 ppm. The intensity values of all spectra were saved into one text file for data binning.[18] Two-dimensional NMR spectra were acquired on a 900-MHz Bruker Avance spectrometer with a cryogenic triple resonance probe.[18]

Data Analysis. The 0.4−3.23, 3.37−4.60, and 5.00−10.0 ppm regions were integrated for data analysis. The omitted regions are from either water (4.60−5.00 ppm) or methanol (3.23−3.37 ppm) due to their irregular behavior. To compensate for fine differences in individual data, binning was performed with a 0.04 ppm interval. The intensity normalization to TSP and conversion of the resulting data were performed using an in-house built Perl program. Statistical analysis employed the following software: Matlab (MathWorks, Natick, MA, USA), SIMCA-P, version 11.0 (Umetrics, Umeå, Sweden), Chenomx (Chenomx, Alberta, Canada), and Excel (Microsoft, WA, USA). A detailed description of the statistical methods was reported previously.[19−22] Specifically, all the data were pareto-scaled and mean-centered. Each class (countries of origin) was assigned a dummy variable, and partial least squares-discrimination analysis (PLS-DA) models were built such that the modeled components can maximize the difference between the scores of each group. The model fitting was carried out until the prediction reliability of the modeled components (expressed as the Q-squared) stops to increase. As orthogonal projections to latent structure-discriminant analysis (OPLS-DA) incorporates an additional step to separate class−orthogonal variations and provides more straightforward interpretation, it was used for two-class cases (i.e., identical species in two different countries). An S-plot was constructed using the modeled correlation and covariation obtained from the OPLS-DA model. Signals with high values in both criteria were selected as markers. It was due to the fact that the correlation reflects the contribution to the class differentiation and that the covariance is related to the signal intensity. The statistical validations of the obtained model were performed using Y-scrambling. It involves a random permutation of the Y variable (class membership) and the subsequent calculation of the classification model and associated evaluation parameters. If the original classification model were due to simple chance, the random permutation will not give any poorer classifications, in terms of R^2 and Q^2, which correspond to the goodness of fit and prediction reliability of the model, respectively. The practical validation of the classification model was conducted by predicting unknown samples (prediction set) on the basis of the data set used to build the model (training set).

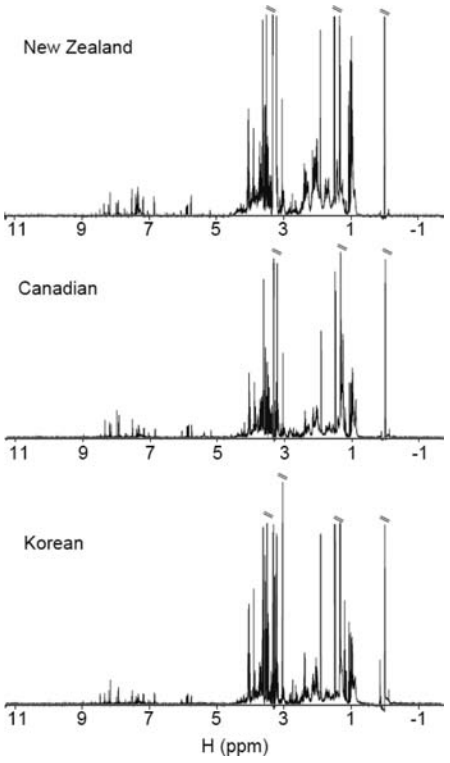

Figure 2. Representative NMR spectra of deer antler samples collected from Canada, New Zealand, and Korea.

RESULTS AND DISCUSSION

Genomic Approach. We analyzed the DNA sequences of 101 deer antler samples collected from Canada, New Zealand, and Korea. The deer species for all of the samples were determined by DNA sequencing of the base pairs in the 439−450 sequence region of the D-loop of the mitochondrial DNA (Figure 1B). Among the 40 Canadian samples, 24 were found to be *C. e. nelsoni*, 13 *C. e. manitobensis*, and 3 *C. e. canadensis* species (Supporting Information, Table S1). Among the 30 New Zealand samples, 27 were *C. elaphus* species (25 samples showed 100% sequence homology; 2 samples 97%. Hereafter, by *C. elaphus*, we mean red deer, which is a subspecies of the *C. elaphus* species, as in ref 23), the remaining 3 samples belonging to *C. e. nelsoni*, *C. e. macneilli* (92% sequence homology), and *C. e. barbarous* (96%) species. Among the 31 Korean samples, 10 belonged to *C. elaphus* species, 11 to *C. e. canadensis*, and 10 to *C. e. nelsoni* species. Therefore, *C. e. nelsoni* was the predominant Canadian species but only a very minor one in New Zealand. Conversely, the predominant New Zealand species, *C. elaphus*, was not found among the Canadian samples. These two species, *C. e. nelsoni* and *C. elaphus*, were almost equally prevalent in the Korean samples. The data indicated also that DNA sequencing (genomic approach) could effectively identify the origins of some species (for example, *C. e. manitobensis*) present only in Canadian samples or exclude Canada as the origin of *C. elaphus*. However, it could not differentiate the country of origin for all of the samples, even though the read-out of the DNA sequence itself is almost unambiguous.

NMR-Based Metabolomic Approach. As the genomic approach alone did not provide information sufficient to reveal the origins of all of the antler samples, we employed the metabolomic

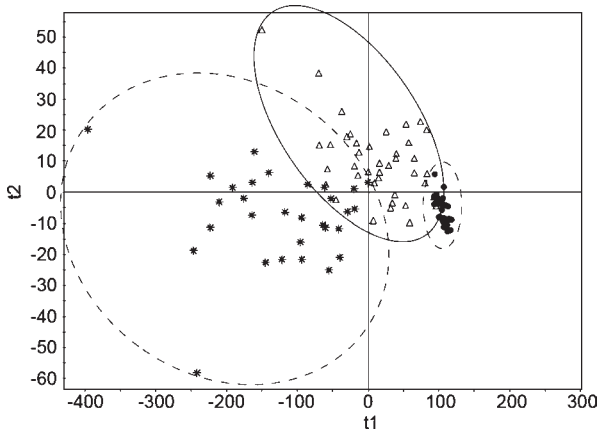

Figure 3. Partial least-squares-discrimination analysis (PLS-DA) score plot. PLS-DA score plots of Canadian (△), Korean (●), and New Zealand (*) samples. The PLS-DA model was obtained with 3 principle components.

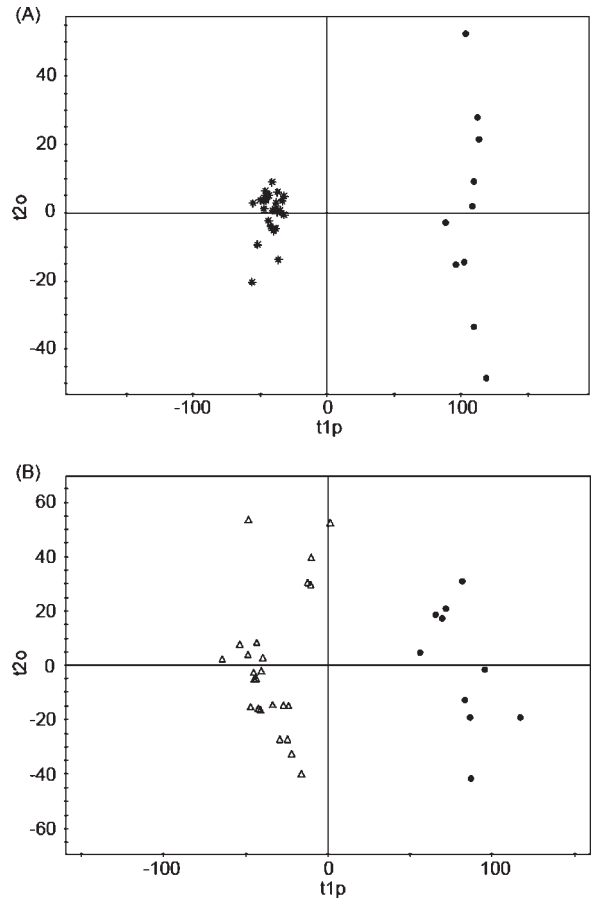

Figure 4. Orthogonal projections to the latent structure-discriminant analysis (OPLS-DA) score plot. New Zealand and Korean samples of *C. elaphus* species (A); Canadian and Korean samples of *C. e. nelsoni* species (B). (A) *, New Zealand samples; ●, Korean samples. (B) △, Canadian samples; ●, Korean samples.

approach to address the environmental or growth conditions of the deer. By this approach, specifically, we analyzed NMR spectra obtained from antler extracts (Figure 2). The spectra, in the 3−4 ppm regions, featured many signals from sugar-containing

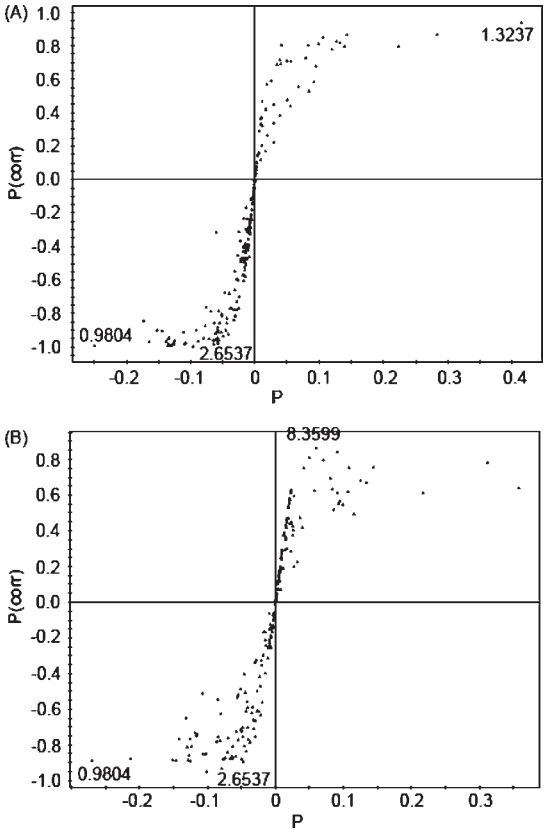

Figure 5. Signals contributing to the differentiation of the samples from the different origins of the same species. (A) S-plot from the OPLS-DA model for the New Zealand and Korean samples of *C. elaphus* species and (B) S-plot from the OPLS-DA model for the Canadian and Korean samples of *C. e. nelsoni* species.

compounds as well as those from methyl groups, probably from branched amino acids.[18] Although the representative spectra of each country's samples seemingly differed, they could not resolve the question of intragroup variation. Therefore, we further performed a multivariate statistical analysis of the entire NMR data set. We applied a partial least squares-discrimination analysis (PLS-DA) to determine if the metabolic profiles could be used to differentiate the origins and find specific signals belonging to each country group (Figure 3). The results showed that in fact, the PLS-DA model could reliably differentiate New Zealand antlers from Korean ones. However, the Canadian samples exhibited some overlap with both New Zealand and Korean samples. The quite tight clustering of the Korean samples might reflect the similar growth conditions in that country, which is much smaller than the other two. Overall, the metabolomic approach, though in some ways very effective, is inadequate for differentiating all samples at once. The encouraging data from a previous report on origin differentiation by NMR-based metabolomics might have been skewed by the much smaller sample sizes for each country.[11]

Combined Genomic−Metabolomic Approach. As both of the approaches showed at least some utility in discriminating antler origins, we reasoned that a combined genomic−metabolomic strategy might effect a significant improvement. First, we applied the genomic approach, without further experimental analysis, to the differentiation of species present only in one country, for example, *C. e. manitobensis* in Canada or *C. e* .

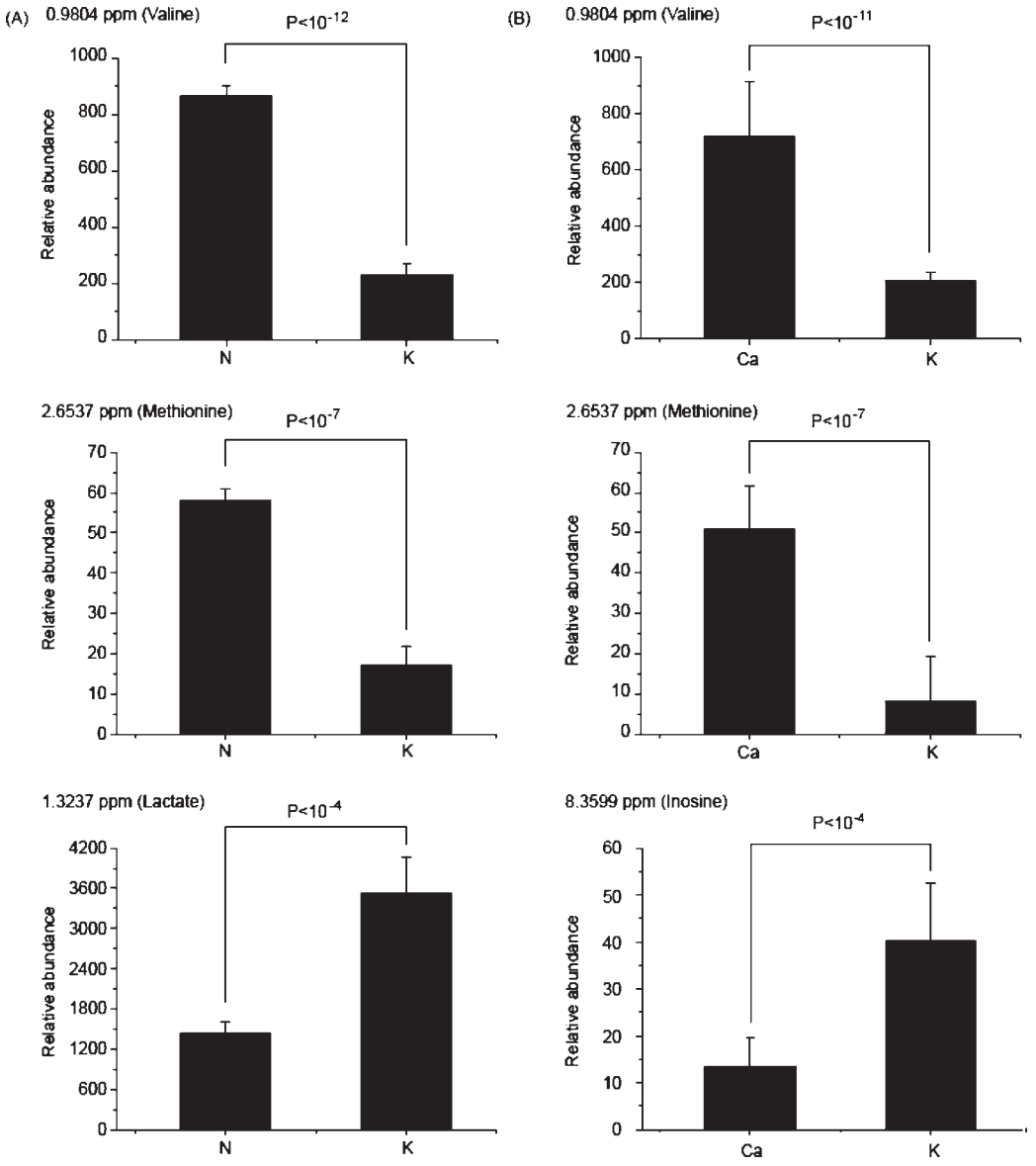

Figure 6. Student's *t*-test for the relative amount of the contributing signals for samples from different origins of the same species. Ca, N, and K represent the samples of Canadian, New Zealand, and Korean origins, respectively. Student's *t*-test graph for New Zealand and Korean samples of *C. elaphus* species (A) and Canadian and Korean samples of *C. e. nelsoni* species (B). (A) Top, 0.9804 ppm (valine); middle, 2.6537 ppm (methionine); bottom, 1.3237 ppm (lactate). (B) Top, 0.9804 ppm (valine); middle, 2.6537 ppm (methionine); bottom, 8.3599 ppm (inosine).

macneilli (92% sequence homology) and *C. e. barbarus* (96%) in New Zealand. Second, we used metabolomics for the species present in large numbers in more than one country. We analyzed *C. elaphus* species, which is found in both New Zealand (25 samples) and Korea (10 samples), and *C. e. nelsoni* species, which is prevalent in both Canada (24 samples) and Korea (10 samples) (see Supporting Information, Table S1). We performed OPLS-DA multivariate analysis on the NMR metabolic profile data for each species. The differentiations were achieved with one predictive component and one orthogonal component for *C. elaphus* species and one predictive component and two orthogonal components for *C. e. nelsoni* species. An overall goodness of fit, $R^2(Y)$, of 98.9% and an overall cross-validation coefficient, $Q^2(Y)$, of 96.9% were obtained for *C. elaphus*, and an $R^2(Y)$ of 91.4% and a $Q^2(Y)$ of 83.8% for *C. e. nelsoni* were also obtained. The resulting models showed that the origins of each species could be clearly differentiated (Figure 4A and B). These results proved to demonstrate that categorizing species by DNA sequencing first and then analyzing the identical species (i.e., those that could not be differentiated genetically) by means of a metabolomic approach can comprehensively discriminate the origins of antler samples.

Statistical Validation. To eliminate any likelihood that clear separations might have occurred by chance, we performed statistical validation using Y-scrambling.[16,17,19] We randomly permutated the *Y* variable value for 200 rounds to rebuild and analyze. We observed a substantial decrease in both R^2 and Q^2 parameters for each model (Supporting Information, Figure S1A and B), the extrapolated value of the Q^2 regression line being about −0.2 or −0.3, respectively.

Marker Compound Identification and Verification. To obtain an idea of which metabolites contributed to the

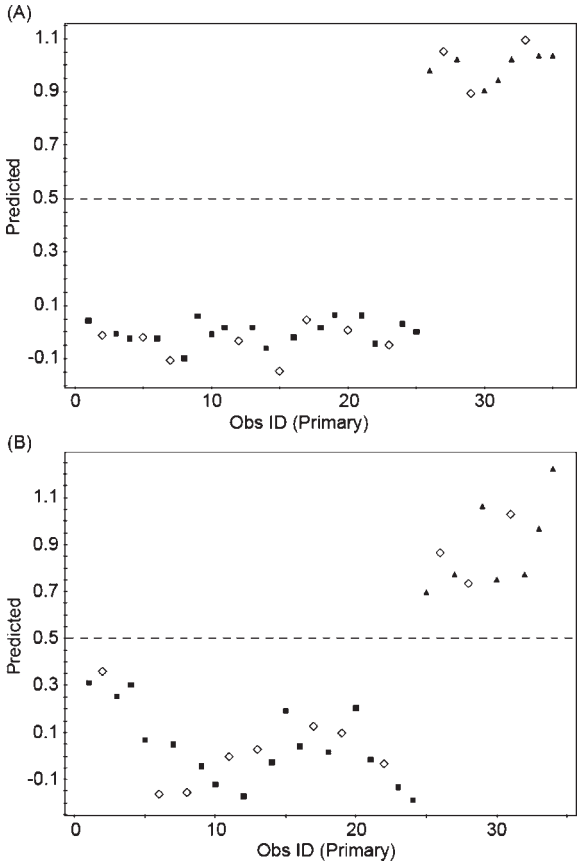

Figure 7. Validation of the statistical model through the prediction of unknown samples from New Zealand and Korean samples of *C. elaphus* species or Canadian and Korean samples of *C. e. nelsoni* species, respectively. (A) Prediction model of *C. elaphus* species: ■, New Zealand samples (training set); ▲, Korean samples (training set). (B) Prediction model of *C. e. nelsoni* species: ■, Canadian samples (training set); ▲, Korean samples (training set). ◊, results for the blind test set. The dashed line represents the a priori cutoff (0.5) for the class membership differentiation. The *X*-axis title Obs ID (primary) means the sample ID used in the study.

differentiation of the same species grown in different countries, we constructed S-plots based on the two OPLS-DA models of each species (see Figure 5A and B). Regarding the *C. elaphus* samples from New Zealand and Korea, the signals at 2.6537 and 0.9804 ppm were higher in the New Zealand cases, whereas the 1.3237 ppm signal was higher in the Korean samples. For the *C. e. nelsoni* samples from Canada and Korea, the same signals (2.6537 and 0.9804 ppm) were higher in Canadian samples, whereas the 8.3599 ppm signal was higher in Korean samples. On the basis of comparisons with the standard samples and two-dimensional spectral analysis (HMBC, DQF-COSY, TOCSY, and HSQC),[18] we identified those signals as coming from methionine (2.6537 ppm), valine (0.9804 ppm), lactate (1.3237 ppm), and inosine (8.3599 ppm). To further test for the biased presence of the marker metabolites, we built a plot with the intensities of their signals in the New Zealand, Canadian, and Korean samples using an independent Student's *t*-test (Figure 6). The result confirmed that these metabolites were significantly biased in one of the groups, contributing to the separation.

For the identified marker compounds, it was interesting to see that the same aliphatic amino acids, methionine and valine, were higher in both the New Zealand and Canadian samples than in the Korean samples, regardless of species. In comparison, maleate and lactate, common organic acids, were higher in the Korean samples than in those from the other two countries. We recently reported that feeding conditions can affect the metabolites detected in deer antlers.[18] It is therefore likely that the differential metabolic profiles of single species antlers from deer living in different countries reflect the growth, food, and environmental differences in those countries. Moreover, our results confirmed that identical species can be differentiated on the basis of their metabolic profiles.

Prediction of Origins for Single Species. An important practical consideration in differentiating the origins of natural products, including deer antlers, is whether a given method can correctly predict unknown samples. As our genomic test (DNA sequencing) could deliver unambiguous species differentiation, we tested our metabolomic model to see if it could predict the origins of unknown samples from a single species. We randomly removed as many as 30% of the samples from the entire data set (test set) and carried out the prediction test with the obtained OPLS-DA model. Therefore, the test set can be considered unknown samples for prediction. Specifically, we removed a total of 11 samples (8 New Zealand and 3 Korean) representing the *C. elaphus* species, along with 11 samples (8 Canadian and 3 Korean) representing the *C. e. nelsoni* species. As shown in Figure 7A and B, all 11 of the *C. elaphus* samples and all 11 of the *C. e. nelsoni* samples were predicted correctly using an *a priori* cutoff value of 0.5, thereby confirming the robustness of the metabolomic antler differentiation model for the same species.

Significance and Future Applications. Natural and agricultural products including animal and herbal varieties, used very widely as foods and dietary supplements, are important economic commodities in all countries. The values of these commodities vary significantly according to their origins. Therefore, correct determination of origins is important not only for accurate economic valuation but also for quality control. For products obtained from species differentially present in different countries, the genomic approach can provide a very reliable means of identification. However, this approach cannot be applied to the differentiation of identical species from different countries. This limitation is particularly important, in that seeds for herbal products are traded internationally more often and more widely than ever before, increasing the likelihood of the same species being grown in various countries. Indeed, some herbal species purposefully are grown in other-than-native countries where cultivation costs are significantly lower. In these cases, differing cultivation techniques and environmental conditions may well lead to a wide range of product qualities. As we showed here, the metabolomic approach can help differentiate origins under those difficult circumstances. Although the metabolomic approach alone could not differentiate all three origins for all of the deer antler samples, it was reliably effective in differentiating identical species samples from different countries. Thus far, many metabolomic studies, including ours, have reported origin differentiation by means of either NMR or mass spectroscopic methods.[16,19,20,24−26] Still, examples of single species origin discrimination, confirmed by DNA sequencing, are hardly seen in the literature. We suggest that the combined genomic−metabolomic approach can improve the reliability of natural product origin differentiation.

■ ASSOCIATED CONTENT

Supporting Information. Statistical validation of the OPLS-DA analysis result by Y-scrambling and classification of species of deer antlers collected from Canada, New Zealand, and Korea using DNA analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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■ ABBREVIATIONS USED

PCA, principal component analysis; PLS-DA, partial least squares-discrimination analysis; OPLS-DA, orthogonal projections to latent structure-discriminant analysis; HMBC, heteronuclear multiple bond coherence; DQF-COSY, double-quantum-filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; HSQC, homonuclear multiple quantum coherence; NMR, nuclear magnetic resonance; RFLP, restriction fragment length polymorphism; TMSP, trimethylsilylpropionic acid sodium salt-d_4.

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