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# Diversity of Kale (*Brassica oleracea* var. *sabellica*): Glucosinolate Content and Phylogenetic Relationships

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Supporting Information

**ABSTRACT:** Recently, kale has become popular due to nutritive components beneficial for human health. It is an important source of phytochemicals such as glucosinolates that trigger associated cancer-preventive activity. However, nutritional value varies among glucosinolates and among cultivars. Here, we start a systematic determination of the content of five glucosinolates in 25 kale varieties and 11 non-kale *Brassica oleracea* cultivars by HPLC-DAD-ESI-MS$^n$ and compare the profiles with results from the analysis of SNPs derived from a KASP genotyping assay. Our results demonstrate that the glucosinolate levels differ markedly among varieties of different origin. Comparison of the phytochemical data with phylogenetic relationships revealed that the common name kale refers to at least three different groups. German, American, and Italian kales differ morphologically and phytochemically. Landraces do not show outstanding glucosinolate levels. Our results demonstrate the diversity of kale and the importance of preserving a broad genepool for future breeding purposes.

**KEYWORDS:** *kale, cabbage, glucosinolates, HPLC-ESI-MS, phylogenetic relationships, KASP assay, SNPs*

## INTRODUCTION

*Brassica oleracea* L. is economically one of the most important species of the Brassicaceae family because cabbage is a widely consumed vegetable all over the world. Worldwide production is 70 million tons.[1] The major reason for this success is large variation in morphology and use by humans of this vegetable. The morphological variation ranges from white and red cabbage over broccoli, cauliflower, kale, kohlrabi, Brussels sprout to lesser known varieties. All domesticated cabbage varieties are believed to have originated from wild cabbage (*B. oleracea* var. *oleracea*) native to different coastal habitats of the Mediterranean and Atlantic.[2] The presently known cabbage cultivars are likely to have originated independently in different regions of Europe from various wild forms. Romans already cultivated several different kinds of cabbage.[3] Evidence for this hypothesis is given by the study of glucosinolates, for example.[4] Song et al. suggested that ancient forms of *B. oleracea* may be thousand-head kale and Chinese kale/kai-lan.[5] The most recent study on the phylogeny of *Brassica* reported *B. oleracea* to be phylogenetically close to *Brassica juncea* (L.) Czern. (Indian mustard), *Brassica incana* Ten. (Mediterranean mustard), *Brassica rapa* L. (turnip rape), and *Brassica napus* L. (rapeseed, hybrid of wild cabbage and turnip rape), as well as *Brassica cretica* Lam. and *Brassica montana* DC. (other wild cabbage types).[6] Surprisingly little, however, is known about relationships of varieties within *B. oleracea*. Louarn et al. demonstrated polyphyly of white cabbage using amplified fragment-length polymorphism (AFLP) but did not resolve relationships among varieties.[7] In contrast, Mei et al. resolved broccoli as early branching in *B. oleracea* and sister-group relationships of kohlrabi and savoy cabbage, as well as white cabbage and Chinese kale, but sampling with *B. oleracea* was too small to allow further inferences.[8] Including all varieties is a huge task given the enormous diversity in the species.

Here, we focus on kale (*B. oleracea* var. *sabellica* L.), which is a valuable fresh vegetable during wintertime given its tolerance against frost. It is mostly assumed to have originated from wild cabbage occurring on cliffs and rocky coasts along the Atlantic, but this is still under dispute.[2] Typical German kale differs from other cabbages in their curly leaves. However, the term kale is rather widely used for different morphological types of large-leafed *B. oleracea* varying in leaf morphology and sometimes described as different varieties, for example. In general, kale is characterized by high levels of various nutrients and further constitutional metabolites such as glucosinolates, flavonoids, and carotenoids.[9] In this study we investigate different kale varieties from various origins and countries (for example, northern Germany, Italy, United States) that differ in traits such as growth height, shape, leaf color, and curling. However, what is not known is whether these geographical origins also form cohesive phytochemical and phylogenetic groups. The main question is, therefore, whether kale constitutes a homogeneous group or whether regional groups of kale can be distinguished phytochemically and genetically.

The phytochemical analysis focuses on five frequently occurring glucosinolates in the leaves in late autumn. Glucosinolates are a large group of anionic, hydrophilic, sulfur-containing secondary plant metabolites. They make a significant contribution to the specific spicy cabbage flavor and bitter taste in *Brassica* crops.[10] More than 100 different

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**Table 1. Glucosinolates Analyzed in This Study, with Their Relevant Characteristics**[a]

| | | $m/z$ [M − H] | | | | |
|---|---|---|---|---|---|---|
| group | trivial name | exptl | theor | error (ppm) | molecular formula | retention time (min) |
| aliphatic | gluconapin | 372.0399 | 372.0418 | 4.9 | $C_{11}H_{19}N_1O_9S_2$ | 7.0 |
| | progoitrin | 388.0368 | 388.0366 | 2.5 | $C_{11}H_{19}N_1O_{10}S_2$ | 4.4 |
| | glucoraphanin | 436.0396 | 436.0400 | 3.5 | $C_{12}H_{23}N_1O_{10}S_3$ | 4.3 |
| aromatic | gluconasturtiin | 422.0578 | 422.0574 | 1.7 | $C_{15}H_{21}N_1O_9S_2$ | 17.0 |
| indole | glucobrassicin | 447.0496 | 447.0537 | 2.6 | $C_{16}H_{20}N_2O_9S_2$ | 15.8 |

[a]Molecular formulas were obtained from The Royal Society Of Chemistry.[52]

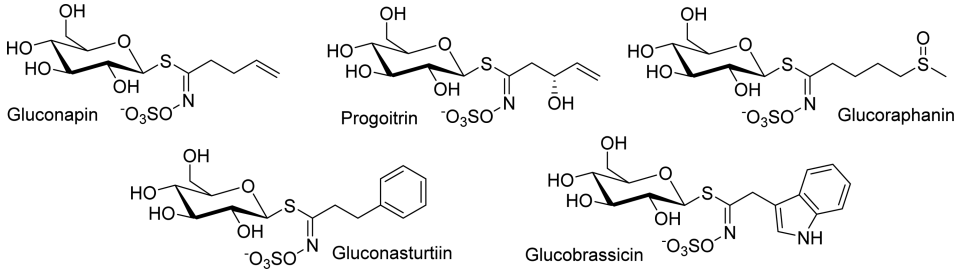


**Figure 1.** Chemical structures of quantified glucosinolates. The structures were obtained by using ChemDraw software (v15.0.0.106, PerkinElmer Informatics, Boston, MA, USA).

glucosinolates are known,[11] and about 20 are predominant in *Brassica* sp.[12] They are derived from amino acids and can be grouped into aliphatic, aromatic, and indole glucosinolates. The endogenous enzyme myrosinase (thioglucoside glucohydrolase) catalyzes the hydrolysis of glucosinolates to yield glucose, sulfate, and aglycones. The aglycones then decompose to breakdown products such as isothiocyanates, thiocyanates, indoles, and organic cyanides (nitriles), depending on the substrate, pH, and availability of certain ions.[13−15] Several isothiocyanates are found to exhibit anticarcinogenic effects against various types of cancer, such as breast, lung, and colon cancer.[16,17] Compounds are bioavailable and show interference in the early stages of cancer development by inducing detoxification enzymes (phase II enzymes), inhibiting activation enzymes (phase I enzymes), and triggering cell cycle arrest or apoptosis.[18−21] The occurrence and differences in specific glucosinolates among various *Brassica* cultivars are therefore an important aspect of *Brassica* breeding. We, here, investigate quantitatively the occurrence of five glucosinolates, that is, the aliphatics gluconapin, progoitrin, and glucoraphanin, the aromatic gluconasturtiin, and the indole glucobrassicin (Table 1; Figure 1). Most of these glucosinolates lead to metabolites that are associated with beneficial anticarcinogenic properties (especially glucoraphanin[22]). Additionally, progoitrin and the aromatic gluconasturtiin contribute to the bitterness of cabbage.[13,23] Progoitrin in particular is associated with the detrimental effect of potentially causing goiter (through the hydrolytic product oxazolidine-2-thione).[24] It is generally assumed that bitter compounds are eliminated by breeding from commercially cultured plants. Therefore, we hypothesized that landraces have increased levels of these compounds compared with commercial cultivars. Glucosinolate profiles are known to differ among *B. oleracea* cultivars.[25] Information on a broad sample of kale cultivars and the determination of specific glucosinolate profiles for certain groups of cultivars may therefore be beneficial for future breeding and choice of kale types for improved nutrition. Whereas glucosinolate levels are known to be influenced by various factors such as life cycle, temperature, and fertilization, a common origin is still considered a crucial aspect of phytochemical profiles.[12]

For investigating relationships between different kales and their relationship to other cabbage cultivars, we made use of the single-plex SNP genotyping platform KASP (Kompetitive Allele Specific Polymerase Chain Reaction (PCR)). Genetic variation is measured on the basis of single-nucleotide polymorphisms (SNPs). They are useful for investigating genetic diversity and discriminating between cultivars and have been commonly applied in research on *Brassica* crops such as *B. napus* (e.g., Dalton-Morgan et al.[26]). SNPs are one of the most common types of genetic variation in eukaryotic genomes and most commonly found in the DNA between genes. The advantages of this assay are low average genotyping errors and cheap genotyping costs, as well as scalable flexibility in applications.[27]

Here, we use SNP and phytochemical data with the aim of identifying glucosinolate profiles specific to certain kale varieties. We hypothesize that different groups of kale have evolved in different parts of the world, differing in their ability to synthesize different glucosinolates. Furthermore, similarities between phytochemical profiles and genetic data allow us to infer the relationships of kale varieties, especially the origin of the American kale varieties.

## MATERIALS AND METHODS

**Plant Material.** In March 2014, seeds of 25 varieties of kale (Table 2) as well as 11 non-kale *B. oleracea* cultivars were seeded in a greenhouse under natural daylight. When plants reached the five-leaf stage, they were transplanted in a randomized scheme into a field at the University of Oldenburg botanical garden. Water and fertilizer were applied according to standard cultural practices. Leaves were harvested in August/September 2014.

**Quantification of the Glucosinolate Content.** *Extraction from Plant Material.* For each kale variety, 10 g of fresh material of the youngest fully developed leaves was weighed (excluding the thick midnerves), treated at 120 °C for 2 h[15] (to inactivate the enzyme myrosinase), ground to a fine powder using a mixer mill, transferred to a screw-top vial, and mixed with 8 mL of 70% v/v methanol/water (of HPLC grade). The sealed vials were sonicated and then stored at −20


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**Table 2. Summary of Kale and Cabbage Varieties Used in This Study**[a]

| variety | included in glucosinolate analysis | included in phylogenetic analyses | seed origin |
|---|---|---|---|
| **German Kale Varieties (*B. oleracea* convar. *acephala* var. *sabellica*)** | | | |
| Lerchenzungen | x | x | Kiepenkerl, Bruno Nebelung GmbH, Everswinkel, Germany |
| Vitessa | | x | N. L. Chrestensen Erfurter Samen- und Pflanzenzucht GmbH, Erfurt, Germany |
| Vitessa | x | | Centrala Nasienna, Saatbau Polska Sp. z o.o., Środa Śląska, Poland |
| Lage Fijngekrulde | x | | N. L. Chrestensen Erfurter Samen- and Pflanzenzucht GmbH, Erfurt, Germany |
| Niedriger Grüner Krauser | x | | PNOS (Polskie Nasiennictwo Ogrodnictwo Szkółkarstwo Sp. z o.o.), Ożarów Mazowiecki, Poland |
| Reflex F1 | x | x | Dürr Samen S. Schwenk e.K., Reutlingen, Germany |
| Redbor F1 | x | x | Kiepenkerl, Bruno Nebelung GmbH, Everswinkel, Germany |
| Frostara | x | x | Kiepenkerl, Bruno Nebelung GmbH, Everswinkel, Germany |
| Winnetou F1 | x | x | Kiepenkerl, Bruno Nebelung GmbH, Everswinkel, Germany |
| Flower-Sprout 'Petit Posy Mix' (Brussels sprouts × kale) | x | x | Gärtner Pötschke GmbH, Kaarst, Germany |
| **Italian Varieties** | | | |
| Black Tuscany | x | x | Thompson & Morgan (UK) Ltd., Ipswich, UK |
| Palmizio Senza Testa | x | x | Thompson & Morgan (UK) Ltd., Ipswich, UK |
| Negro Romano | x | x | Reinhard Lühring, Dreschflegel GbR, Rhauderfehn, Germany |
| **American Varieties** | | | |
| Georgia Southern | x | x | Sustainable Seed Co., Covelo, CA, USA |
| Morris Heading | x | x | |
| Champion | x | x | |
| Vates | x | x | |
| **Local Northern German Kale** | | | |
| Buss Bunde | x | x | Reinhard Lühring, Dreschflegel GbR, Rhauderfehn, Germany |
| Niedriger von Rosenweide | x | x | |
| Rote Palme | x | x | |
| Rote Palme Holterfehn | x | x | |
| Schatteburg | x | x | |
| Jellen | x | x | |
| Neuefehn Hainwatjes | x | x | |
| Lammertsfehn | x | x | |
| Diepholzer Dickstrunk | x | x | |
| **Non-kale Cabbage Cultivars** | | | |
| red cabbage 'Roodkop' *B. oleracea* convar. *capitata* var. *rubra* | x | | Gartenland GmbH Aschersleben, Essen, Germany |
| Savoy 'Vertas 2' *B. oleracea* convar. *capitata* var. *sabauda* | | x | toom, Centor-Warenhandels-GmbH, Köln, Germany |
| butter cabbage *B. oleracea* var. *costata* | x | | Magic Garden Seeds, Andreas Fái-Pozsár, Regensburg, Germany |
| kohlrabi 'Delikateß blauer' (blue) *B. oleracea* convar. *acephala* var. *gongylodes* | | x | N. L. Chrestensen Erfurter Samen- und Pflanzenzucht GmbH, Erfurt, Germany |
| Romanesco broccoli *B. oleracea* convar. *botrytis* var. *botrytis* | x | | Magic Garden Seeds, Andreas Fái-Pozsár, Regensburg, Germany |
| Brussels sprouts 'Hilds Ideal' *B. oleracea* convar. *gemmifera* var. *gemmifera* | | x | toom, Centor-Warenhandels-GmbH, Köln, Germany |
| marrow-stem kale 'Westfälischer Furchenkohl' *B. oleracea* convar. *acephala* var. *medullosa* | x | x | Kiepenkerl, Bruno Nebelung GmbH, Everswinkel, Germany |
| marrow-stem kale 'Walking Stick' *B. oleracea* convar. *acephala* var. *medullosa* | x | x | Thompson & Morgan (UK) Ltd., Ipswich, UK |
| Galician cabbage *B. oleracea* convar. *acephala* | x | x | Reinhard Lühring, Dreschflegel GbR, Rhauderfehn, Germany |
| wild cabbage 'Helgoländer' *B. oleracea* L. | x | x | Botanical Garden of the University of Oldenburg, Oldenburg, Germany |
| Siberian kale *B. napus* ssp. *pabularia* | x | x | Exotic-Samen Wolfgang Meier, Fürstenwalde, Germany |

[a]The assignment to the analyses is displayed as well as the origin of the seeds (e.g., company the varieties have been obtained from).

°C until further analysis. Two individual plants per variety were sampled.

*Analysis.* Kale leaf extracts were obtained after thermal deactivation of myrosinase as aqueous methanolic extracts. Extracts were profiled using an adapted liquid chromatography–mass spectrometry method (LC-MS) from the literature.[28,29] Five glucosinolates (i.e., glucobrassicin, glucoraphanin, gluconasturtiin, gluconapin, and progoitrin; Figure 1) were detected and quantified with high sensitivity by HPLC-qTOF-MS with negative ion electrospray ionization (ESI). A Bruker impact HD qTOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) coupled to an Agilent 1260 series HPLC system was used (Agilent Technologies, Santa Clara CA, USA), consisting of


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online degaser G1322A, bin pump G1312B, high performance autosampler HiP-ALS G1367E, thermostatted column compartment G1316A, and diode array detector DAD-VL G1315D. Before analysis, 2 mL of the extracted samples was filtered (0.45 $\mu$m pore size) into 2 mL screw-top vials. The HPLC column was a 150 mm × 2 mm (2.8 $\mu$m particle size) Pursuit XRs Ultra 2.8 diphenyl column (Agilent Technologies). The mobile phase was 0.005% formic acid (HCOOH) with an acetonitrile/water gradient ($CH_3CN$) over 75 min at a flow rate of 0.5 mL/min. The gradient profile increased from 5 to 75% linearly in 45 min followed by a return to 5% and 30 min isocratic to re-equilibrate. The eluate absorbance was monitored by an UV detector at 229 nm. MS scans were performed in the negative ion mode on the glucosinolate $[M - H]^-$ ions, and spectra were recorded between $m/z$ (mass-to-charge ratio) 100 and 700 (capillary voltage, 4500 V; nebulizer, 1.8 bar; dry gas temperature, 200 °C; dry gas flow, 9 L/min). For quantification eight-point calibration curves (see Supplementary Figure 1) were obtained for each glucosinolate from authentic reference substances (analytical standard glucosinolates from PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany). Quantification was carried out in the negative ion mode on extracted ion chromatograms of each respective glucosinolate $m/z$ value with isolation width of 0.002 Da. All Pearson correlation coefficients $R^2$ were found to be >0.99 and repeat injections yielding a relative standard deviation (RSD) <5%. The lowest quantifiable amount is indicated for each glucosinolate by the signal-to-noise ratio (S/N) (see Table 3). Mass calibration was carried out using a 0.01 mM sodium formate solution injected prior to each chromatographic run using an enhanced quadratic calibration algorithm. Spiking of the kale extract with authentic reference compounds was carried out with a RSD <5% indicating that no appreciable matrix effect was operating.

**Table 3. Parameters for Calibration Curves: Slope, Axis Intercept, Pearson Correlation Coefficient $R^2$, as well as Relative Standard Deviation of Analytical Repeat Injections (RSD) and Signal-to-Noise Ratio (S/N) of fhe Five Glucosinolate Standards**

| glucosinolate | slope | axis intercept | $R^2$ | RSD (%) | S/N |
|---|---|---|---|---|---|
| gluconapin | 0.0012 | −0.1089 | 0.9998 | 3.97 | 45 |
| progoitrin | 0.0006 | −0.3175 | 0.9980 | 3.01 | 230 |
| glucoraphanin | 0.0039 | 0.0663 | 0.9998 | 4.16 | 45 |
| gluconasturtiin | 0.0005 | −0.0386 | 0.9999 | 4.37 | 195 |
| glucobrassicin | 0.0012 | 0.1321 | 1.0 | 3.23 | 55 |

*Data Evaluation.* For data evaluation, ESI Compass 1.3 Data Analysis software was used (version 4.0.234.0, Bruker Daltonik GmbH, Bremen, Germany). Statistical analyses were conducted using the software R (v3.0.3, R Core Team[30]), specifically analysis of variance (ANOVA), Tukey's HSD test, and Pearson's product-moment correlation analysis.

**Phylogenetic Analyses Using KASP Assay.** *DNA-Extraction and KASP Assay Analysis.* For each variety, one intact, fully developed leaf was cut, placed in a plastic bag with silica beads inside, and stored until it was completely dried. DNA was extracted from leaves using the innuPREP Plant DNA Kit (Analytik Jena AG, Jena, Germany) and afterward stored at −20 °C until further analysis. The subsequent KASP assay, developed by KBioscience/LGC Genomics (Hoddesdon, Hertfordshire, UK), was carried out by TraitGenetics (TraitGenetics GmbH, Gatersleben, Stadt Seeland, Germany). Each sample was analyzed with 100 KASP markers well distributed over the genome of *B. oleracea*. KASP assays rely on allele-specific oligo extension and fluorescence resonance energy transfer (FRET) for signal generation.[31] In the KASP cycle, an allele-specific primer matches a target SNP and, with a common reverse primer, amplifies the target region. In a second PCR round, the reverse primer binds to the previously generated product and thus makes a complementary copy. In a third PCR round, a fluorescence-labeled oligo (FAM or HEX from FRET cassette) binds to the amplicon from the previous round.[32,33]

*Data Analysis.* Phylogenetic relationships were inferred using Bayesian inference (BI) and neighbor-net methods. The BI analysis was conducted using MrBayes (v3.2.4, Ronquist et al.[34]) sampling across the entire general time reversible (GTR) model space, including gamma. Two independent runs of 10,000,000 generations were completed with four chains each (three heated, one cold). Trees were sampled every 100 generations; the first 25% of generations were discarded as burn-in. A majority-rule consensus of the remaining trees from the two runs was produced with posterior probabilities. Siberian kale (*B. napus* var. *pabularia* DC.) was used as outgroup taxon. Compared to a phylogenetic tree, phylogenetic networks can often better illustrate the evolutionary history, for example, in the case of hybridization. They display the extent of conflicting signals in the data.[35] Neighbor-net is a distance-based method for constructing phylogenetic networks, based on the neighbor-joining (NJ) algorithm.[36] A neighbor-net was constructed using SplitsTree (v4.13.1, Huson and Bryant[37]) with uncorrected $P$ distances, and ambiguous states were matched. A detailed description of the underlying mechanism of the method is given by Bryant and Moulton.[38]

## RESULTS

**Quantification of the Glucosinolate Content.** The five glucosinolates previously described in kale, gluconapin, progoitrin, glucoraphanin, gluconasturtiin, and glucobrassicin, were identified in the chromatogram on the basis of the high-resolution $m/z$ value of their $[M - H]$ pseudomolecular ion, which all showed errors <5 ppm. Additionally, the structures were confirmed using a tandem MS measurement with all glucosinolates showing characteristic fragment ions at $m/z$ 259.01 ($C_6H_{12}O_9S$). On the basis of this characteristic fragmentation mechanism, a search for previously unreported glucosinolates was carried out by construction of extracted ion chromatograms in All $MS^n$ mode (see Supplementary Figure 2). Isothiocyanate products (e.g., erucin, sulforaphane) are absent from the LC-MS chromatograms (see Supplementary Figure 4).

The contents of the five glucosinolates were analyzed in different kale and cabbage samples, with two individual plants being measured for each variety with analytical duplicate measurements. The resulting levels, received with the help of reference standard measurements, are summarized in Supplementary Table 1. The gluconapin content varied between 0.005 mg/100 g fresh weight (FW) (Redbor) and 19.6 mg/100 g FW (Vates) (Figure 2). The American varieties (especially Georgia Southern, Champion, and Vates) showed significantly higher gluconapin contents compared to all others ($p < 0.05$; Figure 3A). Furthermore, much gluconapin was contained in wild cabbage (Wildkohl) with 16.44 and 11.12 mg/100 g FW. In the Italian varieties (except one individual of Negro Romano), the German Lerchenzungen and Lage, as well as the Galician cabbage, no gluconapin could be detected at all. The northern German landraces and the non-kale cabbage cultivars had only low gluconapin contents. Both marrow-stem kale Furchenkohl and Walking Stick were similar in their gluconapin contents. Progoitrin content is correlated with the gluconapin content ($R = 0.86$, $p < 0.001$). The content ranged from 0.007 mg/100 g FW (Lerchenzungen) to 13.31 mg/100 g FW (wild cabbage) (Figure 2). Again, the American kale had significantly more progoitrin than the others ($p < 0.05$; Figure 3B). Most samples exhibited rather low progoitrin; only wild cabbage, Frostara, and one individual of Flower-Sprout had higher contents. Glucoraphanin values ranged from 0.09 mg/100 g FW (Palmizio) to 91.06 mg/100 g FW (Romanesco) (Figure 2). There were only a few varieties that consistently contained


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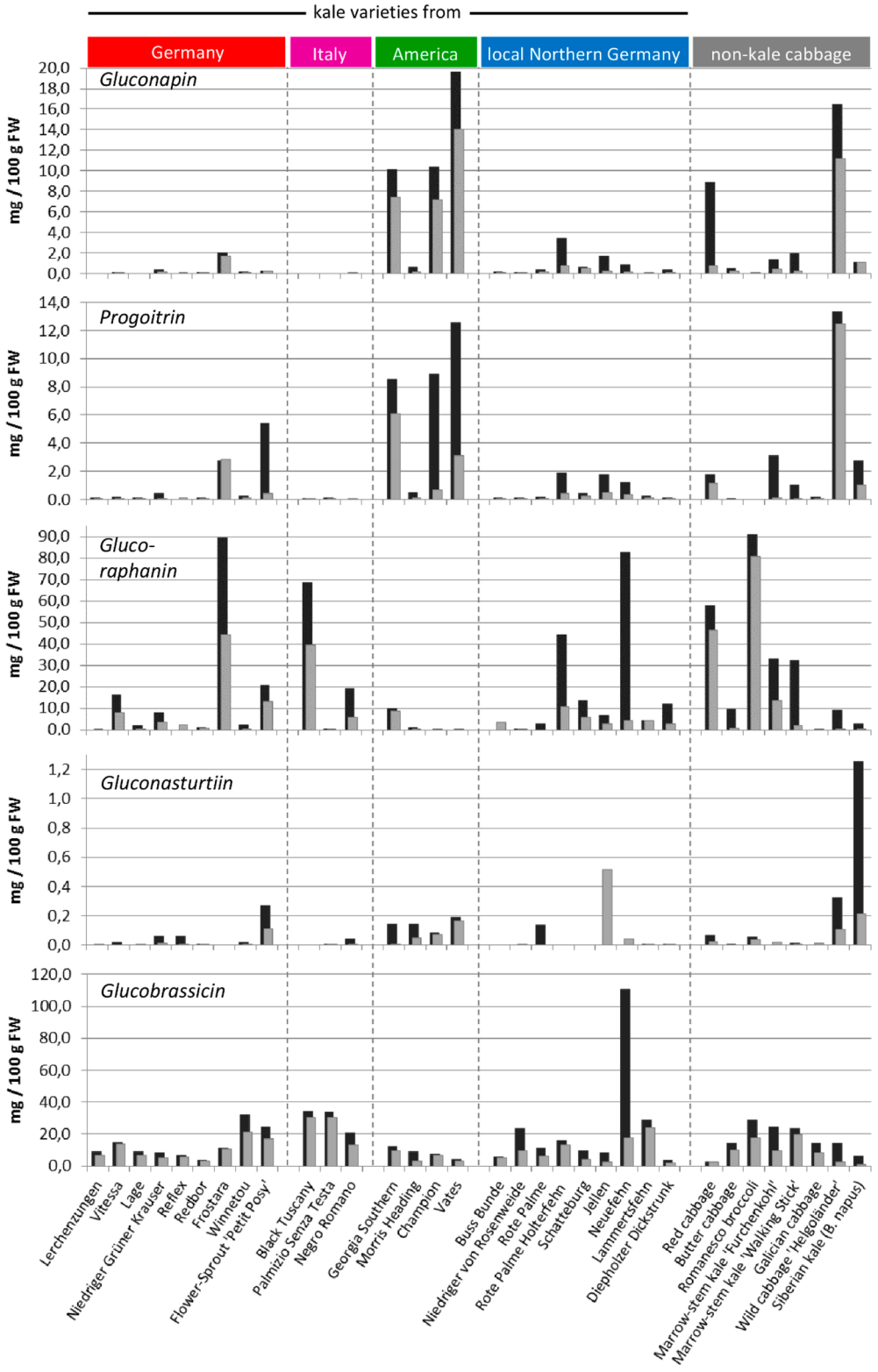


**Figure 2.** Glucosinolate content (mg/100 g FW) of different kale and cabbage varieties. Two individual plants per variety were measured (black and gray bars). Note the different scales.

much glucoraphanin (Frostara, Black Tuscany, red cabbage, and Romanesco). In German kales mostly low glucoraphanin levels had been found (in the range of approximately 1−2 mg/100 g FW), with the exception of Frostara (89.47 and 44.22 mg/100 g FW) and Flower-Sprout (20.66 and 12.99 mg/100 g FW). More glucoraphanin has been detected in the Italian Black Tuscany (68.69 and 39.66 mg/100 g FW). American varieties rarely contained glucoraphanin. The northern German landraces had relatively low amounts as well (below 10 mg/100 g FW); striking outliers were Holterfehn and Neuefehn, which showed big differences between the two individuals (in Neuefehn nearly 20 times higher). There were no significant


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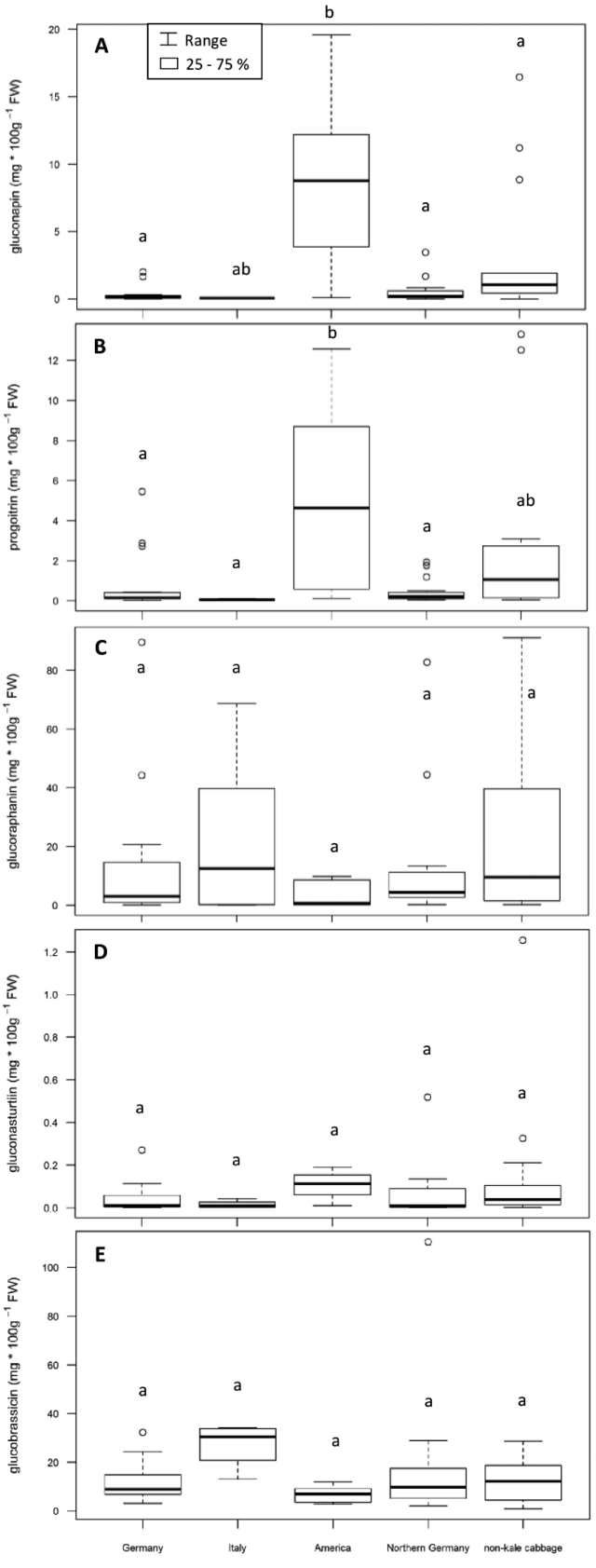


**Figure 3.** Differences in glucosinolate content (mg/100 g FW) between the groups of kale and cabbage varieties: (A) gluconapin content; (B) progoitrin content; (C) glucoraphanin content; (D) gluconasturtiin content; (E) glucobrassicin content. Note the different scales. Different letters above each box plot indicate significant differences in a post hoc Tukey's HSD test ($p < 0.05$).

differences between the sample groups ($p = 0.33$; Figure 3C). Gluconasturtiin was the glucosinolate that was contained in all samples in very low amounts, between 0.002 mg/100 g FW (several varieties) and 1.26 mg/100 g FW (Siberian) (Figure 2). There were many varieties in which gluconasturtiin could not be detected at all. Elevated levels were found only in Flower-Sprout (0.27 and 0.11 mg/100 g FW), the American kale (approximately 0.1 mg/100 g FW), and the wild cabbage (0.33 and 0.11 mg/100 g FW). The Italian and northern German varieties and the non-kale cabbages contained only traces of gluconasturtiin. There were no significant differences between the sample groups ($p = 0.58$; Figure 3D).

The only glucosinolate that has been found in all varieties without exception was glucobrassicin. The detected amounts ranged from 0.85 mg/100 g FW (Siberian) to 110.48 mg/100 g FW (Neuefehn) (Figure 2). All sample groups and individuals were more or less uniform with similar values. Notably high contents had been found in Winnetou and Flower-Sprout (around 20 mg/100 g FW), both Italian varieties Black Tuscany and Palmizio (between 30 and 34 mg/100 g FW), and the northern German kale Lammertsfehn (24 and 29 mg/100 g FW). Furthermore, much glucobrassicin was also contained in Romanesco (28.7 and 17.6 mg/100 g FW) and both marrow-stem kale cultivars (24.7/9.6 and 23.4/19.7 mg/100 g FW, respectively). Strong variation between individuals occurred only in Neuefehn plants. There were no significant differences between the sample groups ($p = 0.1$; Figure 3E).

With regard to the total amount of all five glucosinolates, there were no significant differences between countries of origin ($p > 0.05$). The highest total amount was detected in the northern German kale Neuefehn (195 mg/100 g FW).

**SNP Analysis.** Our phylogenetic analyses identified consistently a group containing all German varieties distinct from the non-kale *B. oleracea*, the wild cabbage, Galician cabbage, Walking Stick, and the American varieties (Figure 4; Supplementary Figure 3). The three Italian varieties form a single cluster located between the two above-mentioned groups. The marrow-stem kales Furchenkohl and Walking Stick do not form an exclusive group. As seen from the Bayesian phylogeny, the American kales do not form a monophyletic group (Supplementary Figure 3). Vates and Champion as well as Georgia Southern and Morris Heading each cluster together.

## DISCUSSION

**Patterns of Glucosinolate Distribution.** On the basis of our analyses, *B. oleracea* is characterized by high glucobrassicin and low gluconasturtiin levels. Glucoraphanin, progoitrin, and gluconapin are mostly low. A similar distribution was found by Ciska et al.,[39] for example, in kale cultivar Halbhoher Grüner Krauser.

Correlations among glucosinolate levels, such as progoitrin and gluconapin in our analysis ($p < 0.001$), can be understood in the light of their biosynthetic pathways. Gluconapin, progoitrin, and glucoraphanin are aliphatic glucosinolates, whereas gluconasturtiin belongs to the aromatics and glucobrassicin to the indoles. High levels of gluconapin and progoitrin are, therefore, indicative of strong diversion of precursors to the aliphatic branch of glucosinolate production with an efficient conversion of glucoraphanin to gluconapin but inefficient further side-chain modifications to progoitrin.[40] Consequently, no other correlations among two glucosinolates of independent biosynthetic pathways were found. Among


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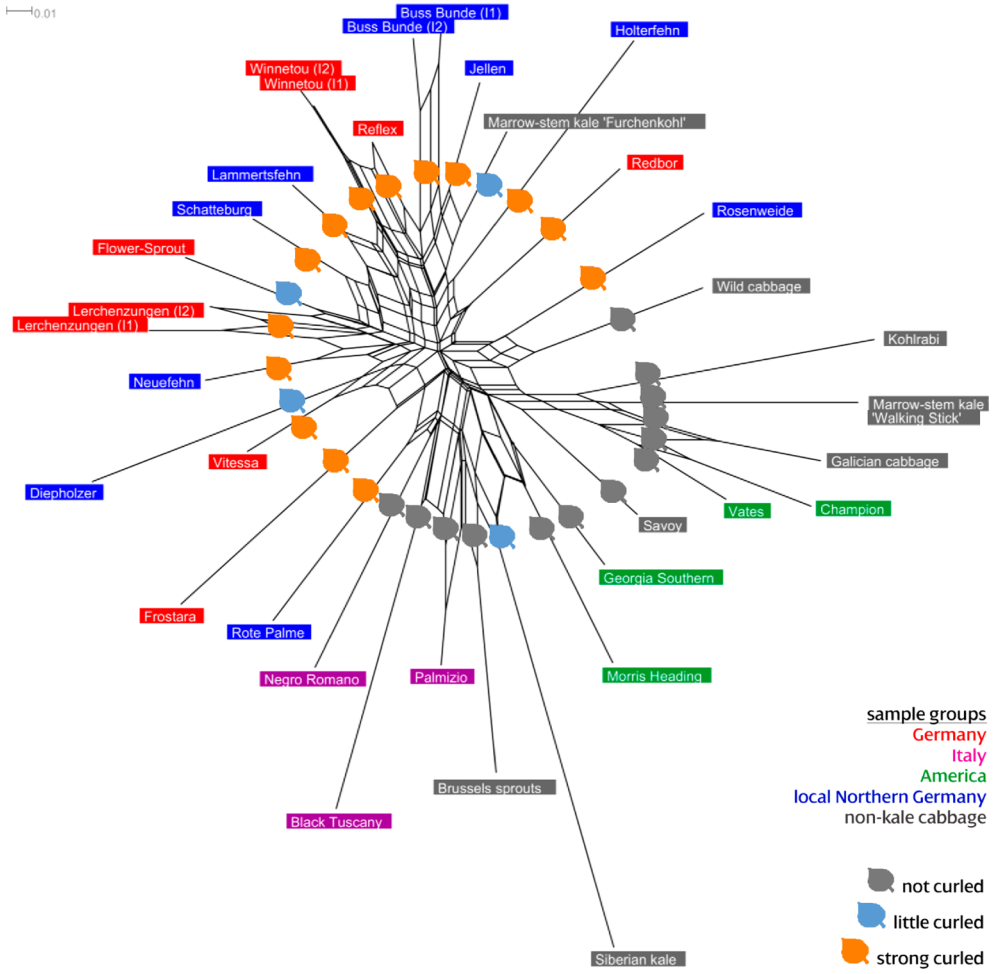


**Figure 4.** Neighbor-net of different kale and cabbage varieties based on the KASP analysis. Sample names are color coded according to their origin. Numbers beside branches are Bayesian posterior probabilities (support > 70) for the respective clade. Leaf margin curliness of the varieties is indicated.

most regional groups of kale varieties, the glucoraphanin content varies substantially. Gluconasturtiin is the glucosinolate that exhibits the lowest contents and in many samples could not be detected at all. Presumably, this is due to an inefficient biosynthetic pathway leading to aromatic glucosinolates in kale. Because glucobrassicin is prevalently found in the genus *Brassica*, it is found in all varieties here. Nilsson et al. found similar proportions in a Swedish kale variety: gluconapin, progoitrin, and gluconasturtiin are often not detectable or present only in traces; glucoraphanin is contained in intermediate levels, whereas indoles such as glucobrassicin and its derivates are most abundant.[25]

We detected at times large variations in biological replicates, hence, variations between the two analyzed individuals. Because the experimental error in analytical replicates is an order of magnitude smaller if compared to these variations, these values must constitute real variations in glucosinolate concentrations in the kale leaves. Therefore, these results should not be interpreted as indicating typical glucosinolate composition of single varieties, for which more individual plants would have to be analyzed per variety. Our intention is to compare groups of kale from different origins. However, it is known that herbivory may increase the amount of secondary metabolites. Mewis et al. as well as Falk et al. demonstrated that herbivores such as slugs and caterpillars attacking plant tissue (leaves) lead to increased aliphatic glucosinolate content used to defend the plants against the animals.[41,42] The content of indole glucosinolates increased slightly, too. One can suppose now that some of the sampled plants likewise were attacked by herbivores and therefore have stress-induced increased glucosinolate levels. That cannot be confirmed for each plant, because they have not been monitored in detail, but we did observe caterpillars on the plants. These were removed to save the plants, but it is quite likely that they had already slightly damaged the plants—and induced glucosinolate production. One Palmizio plant that was extremely affected by herbivores was additionally sampled to compare it with another individual of the same variety with intact leaves. The results demonstrate that the glucosinolate levels in the affected plant are much higher (gluconasturtiin level, 0.01 mg/100g FW (unaffected) → 0.18 mg/100g FW (affected); glucoraphanin, 9.9 → 66.9; progoitrin, 0.01 → 0.06; glucobrassicin, 28.9 → 62.4). However, as seen by the results the rank order among compounds remained almost the same. As far as the production of a particular glucosinolate is concerned, it is still unclear in which variety it is induced, because there are often only one or two glucosinolates, the amounts of which differ between our two measured individuals. High indole glucosinolate contents, for example, may for some varieties also be associated with other stress factors during the cultivation period, such as high temperatures in summer[43,44] or


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the lack of water that enhances the glucosinolate synthesis.[39] However, these should have affected all plants in the same way, although some cultivars may be more susceptible.

**Kale from a Phylogenetic Perspective.** Our analysis is the first to employ KASP assays in a phylogenetic framework using SNPs to infer internal groups in *B. oleracea*. The results demonstrate the general feasibility of our approach by delimiting major groups expected on the basis of morphology and coherent with phytochemistry. Nevertheless, low support values indicate that more SNP markers need to be analyzed in the future.

An advantage of the neighbor-net is that it resolves relationships but at the same time displays uncertainties of the underlying data and possible connections between varieties and groups. On this basis (Figure 4), the distinction of German, American, and Italian kale becomes obvious.

The assumption that the wild cabbage is the ancestor of all cabbage and kale varieties can be confirmed to a wide extent. Although it is not completely basally branching, wild cabbage is near non-kale cultivars such as savoy, Brussels sprouts, and kohlrabi as well as Galician cabbage and the American varieties, which fits with the broader analysis of the *Brassica* phylogeny.[6] There, Brussels sprouts and kohlrabi also stand together, neighbored to marrow-stem kale on a separate branch. In the study of Song et al. the authors demonstrated that palm kale such as Palmizio or Negro Romano is closely related to Brussels sprouts, marrow-stem kale, and broccoli.[5]

Among the non-kale *B. oleracea* cultivars, red cabbage and Romanesco both have an outstanding high glucoraphanin level. For red cabbage, this was found by Ciska et al., too.[39] The other glucosinolates' values are similar as well: much gluconapin and progoitrin and not as much glucobrassicin as in other varieties.

High gluconapin, progoitrin, and gluconasturtiin contents appear mainly in the branches close to wild cabbage and the American varieties (except Morris Heading; Figure 4). High glucobrassicin levels instead are found predominantly in varieties such as Neuefehn, Lammertsfehn, Winnetou, and Black Tuscany, but these are phylogenetically not very close to each other.

Varieties with plain leaves that are not or only little curled (marked in gray and blue) are mainly the kales from America and Italy as well as non-kale cabbage cultivars (such as wild cabbage). This distinguishes them from the German kale varieties with their typically strongly curled leaves (Figure 4).

Our phylogenetic analysis allows for an independent estimate of the relationship of different varieties.

**Characterization of Different Kale Types.** The Italian kales Black Tuscany, Palmizio, and Negro Romano (Negro Romano = Black Roman) are morphologically quite similar. One striking similarity between the three cultivars and distinguishing the Italian kale from the others is a high glucobrassicin content. They also have little gluconapin and progoitrin, and in Black Tuscany we additionally find a high glucoraphanin content. Because glucoraphanin is converted to gluconapin and progoitrin through enzymatic processes,[40] it is imaginable that in this variety the appropriate enzyme is missing or not functioning well. The low progoitrin level and thus reduced bitterness may account for the frequent use of Italian kale's leaves for salads. The Italian kale varieties have been referred to as *B. oleracea* var. *palmifolia* instead of var. *sabellica*,[45] which could be translated as palm kale.[46] Körber-Grohne hypothesized that the origin of kale likely is in Italy or Greece.[2] The phylogenetic position of palm kale near the outgroup in this study would support this assumption.

In the American varieties the highest gluconapin and very high progoitrin contents have been found. In the phylogenetic analysis the varieties are placed near non-kale *B. oleracea* cultivars (savoy, Galician cabbage, kohlrabi) and not far from wild cabbage (Figure 4; Supplementary Figure 3). Because wild cabbage also contains much gluconapin and progoitrin—in contrast to most other varieties—one might hypothesize that the American varieties are more closely related to wild cabbage than to kale varieties. Thus, it seems likely that American kale has been bred in America from wild kale or related types rather than from typical German kale. Song et al. reported Vates to be closely related to savoy cabbage,[5] in agreement with our neighbor-net analysis (Figure 4) and also supported by the leaves of American type kale resembling more those of savoy, marrow-stem kale or butter cabbage than curly kale.

A similar argument can be brought forward for Galician cabbage. Congruent with the position in the neighbor-net, the glucosinolate compositions of Galician cabbage and Walking Stick marrow-stem kales are quite similar. Velasco et al. also studied a local Galician kale variety (named "MBG-BRS0468") with similar results: glucobrassicin is most abundant followed by glucoraphanin and gluconasturtiin; progoitrin and gluconapin were not found.[47] Marrow-stem kales Furchenkohl and Walking Stick and Galician kale are quite unique in their glucosinolate contents. One can suppose that evolutionarily they are closely related to each other, because, moreover, they resemble each other in appearance and leaves. However, looking at the phylogeny, Walking Stick and Galician kale are placed near wild cabbage and the American kales. Furchenkohl, however, is placed distantly near Buss Bunde and other northern German landraces. Körber-Grohne reported the origin of Walking Stick on the island of Jersey and specified it as *B. oleracea* var. *acephala* f. *exaltata*, but no further information on its origin is available.[2]

The northern German landraces have not been involved in extensive domestication or inbreeding selection. However, they do not exhibit significantly higher glucosinolate levels than other kale varieties ($p > 0.05$). Additionally, the general lack of coherence among German landraces and German commercial cultivars indicates multiple origins of German commercial cultivars from various northern German landraces. Holterfehn and Jellen as well as Buss Bunde, Rote Palme, and Diepholzer each have very similar glucosinolate contents. However, looking at the phylogenetic results, none of them are placed in a sister-group relationship. Many of these northern varieties have their native region around the northern German city Leer (Holterfehn, Jellen, Buss Bunde, Lammertsfehn, Schatteburg, Neuefehn). In the BI phylogeny (Supplementary Figure 3) they cluster nearly perfectly together. In the neighbor-net (Figure 4) we find them also quite near each other.

Flower-Sprout, a hybrid between kale and Brussels sprouts, is a newly developed hybrid cultivar originating in England (Gärtner Pötschke GmbH, Kaarst, Germany). It is not known which kale variety was used as parent for hybridization, but with reference to Flower-Sprout's red leaves, it is imaginable that the parental variety also had red leaves (such as Redbor or Rote Palme).

Siberian kale was used as outgroup taxon in phylogenetic analyses because it belongs to *B. napus* ssp. *pabularia*. The leaves of Siberian resemble those of kale a bit, because it is believed that *B. napus* originated from hybridization between


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turnip (*B. rapa*) and kale. Carlson et al. reported high progoitrin and glucobrassicin contents in this variety.[48] We instead found a high gluconasturtiin content, which is consistent with studies on *B. rapa*.[49,50]

**Implications for Health-Oriented Breeding.** Although we have assembled data on different aspects of the glucosinolate content and relationships of kale and demonstrated differences between regional origins, a number of mechanisms may explain why glucosinolate levels differ between varieties. Several sets of genes regulate the content of specific glucosinolates in different cultivars. Also, additional factors may influence the glucosinolate composition (soil properties, drought, temperature, light, etc.[12]). Because some of the glucosinolate metabolites exhibit cancer chemopreventive or even anticarcinogenic properties and others have rather detrimental effects and can decrease food quality through bitter taste (due to high progoitrin levels, for example[23]), there has to be a trade-off between beneficial and unfavorable properties. A combination of different metabolites in plants should be aimed at. Most potent breakdown products in terms of anticarcinogenic effects are the ones of glucoraphanin, such as the isothiocyanate sulforaphane.[13,51] Therefore, kale varieties with elevated glucoraphanin levels are desirable as functional foods with added health benefits. Frostara and Black Tuscany as well as red cabbage and Romanesco produce much glucoraphanin. In comparison, typical broccoli varieties contain around 10 mg glucoraphanin/100 g FW.[28] Our quantitative data show glucoraphanin levels in some kale varieties can be as high as 10 times that of an average broccoli, which is currently the gold standard with respect to provision of glucoraphanin dietary exposure. In contrast, the American varieties, Palmizio and Lerchenzungen, have rather low amounts. Frostara at the same time produces comparatively much progoitrin, and so do Siberian kale, the American kale, and red and wild cabbage. These may therefore taste more bitter. The aromatic gluconasturtiin is mostly present in low amounts in *B. oleracea*; Siberian kale (*B. napus*) instead contains much higher volumes. The wide range of variability in glucosinolate content among kales can be used as information for breeding and developing new varieties with enhanced benefits.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b01000.

Supplementary Figure 1: calibration curves of glucoraphanin and glucobrassicin. Supplementary Figure 2: base peak chromatogram (BPC) and extracted ion chromatograms (EIC) of kale variety Frostara. Supplementary Figure 3: Bayesian Inference phylogeny. Supplementary Figure 4: extracted ion chromatogram (EIC) of sulforaphane and glucoraphanin in kale variety Buss Bunde. Supplementary Table 1: glucosinolate values of analyzed kale and cabbage samples (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

AFLP, amplified fragment-length polymorphism; ANOVA, analysis of variance; BI, Bayesian inference; Da, dalton; ESI, electrospray ionization; FRET, fluorescence resonance energy transfer; FW, fresh weight; GTR, general time reversible; HD, high definition; HPLC, high-pressure liquid chromatography; KASP, "kompetitive" allele-specific polymerase chain reaction; MS, mass spectrometry; *m/z*, mass-to-charge ratio; NJ, neighbor joining; PCR, polymerase chain reaction; ppm, parts per million; qTOF, quadrupole time-of-flight; RSD, relative standard deviation; S/N, signal-to-noise ratio; SNP, single-nucleotide polymorphism

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