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CINNAMON SPECIES VARIATIONS FOR PHYTOCHEMICALS IN TWO DIVERSE HABITATS OF BALI, INDONESIA

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SUMMARY

Cinnamon (*Cinnamomum burmannii* Blume) plant leaf extracts contain phytochemicals and have been used as potential biofungicides against plant pathogenic fungi by inhibiting the growth of their colonies, biomass, and spores, both in vitro and ex vivo. This past study demonstrated that cinnamon leaves differed in phytochemical compounds from one habitat to another. Cinnamon leaves from Belok Sidan Village, Petang Subdistrict, contain flavonoids, steroids, phenolics, tannins, and those from Bedugul Village, Baturiti Subdistrict, contain alkaloids, steroids, phenolics, and saponins. The probing study aimed to unveil whether the phytochemical variations in cinnamons are due to biotic and abiotic factors. The cinnamon species' identification from two distinct habitats engaged two molecular markers, *rbcL* and *mat-K* genes. Comparing both habitats' environmental parameters, i.e., soil, water, minerals, temperature, humidity, wind speed, coordinates, and altitudes, took place. The DNA barcode analysis revealed that the two cinnamon plant types were likely of the same species, namely, *Cinnamomum tamala* or *C. osmophloeum*. Soil and climate analyses indicated contrasting conditions between Petang and Bedugul habitats. The C, N, and K contents were higher in the Bedugul soil than in the Belok Sidan. The soil water contents differed in both locations, especially the percentage of dry air (DA) and field capacity (FC). The two sites also differed in temperature, humidity, wind speed, coordinates, and altitude. This study provides solid evidence that environmental factors highly contribute to the phytochemical variations in cinnamon species.

Keywords: Cinnamon, DNA barcoding, phytochemical diversity, physical factors, soil, species identification

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Key findings: The first reports of Cinnamon species (*Cinnamomum tamala* and *C. osmophloeum*) emanated in Bali, Indonesia. Cinnamon plants collected from two distinct locations have different phytochemical compounds. The variations in phytochemical contents have shown influences by environmental factors.

INTRODUCTION

Cinnamon (*Cinnamomum burmannii* Blume) is a species of wooden tree that can grow in diverse climatic conditions and reach a height of 50 meters. The cinnamon bark can serve as a medicine to cure digestive tract-related disturbances. The bark and its essential oil have a registration with the British and European Phytomedicines, especially useful in tea as an antibacterial and antifungal agent (Dao *et al*., 1999). Cinnamon can also inhibit the in vitro growth of opportunistic pathogenic yeast (*Candida albicans*) (Rachma, 2012). The cinnamon bark extract at 1% and 2% concentrations can inhibit the growth of *Aspergillus flavus* and *Fusarium moniliforme*, with diameter inhibition zones of 27 and 57 mm, respectively.

With these inhibition zone diameters, the cinnamon extract received a very strong inhibitor extract category (Novrianti *et al.,* 2019). Observations from in vitro tests have also shown that cinnamon leaf extract significantly inhibited the fungal colony growth, biomass, and spore formation. The cinnamon leaf extract application at concentrations of 1%, 1.25%, 1.50%, 1.75%, and 2% notably inhibited the growth of *Fusarium oxysporum* f.sp. lycopersici, with inhibition percentages of 41.66%, 78.11%, 88.33%, 91.11%, and 100%, respectively, compared with the control (Darmadi *et al.*, 2015).

The antifungal function of cinnamon bark and leaf extracts could be due to secondary metabolites in the plant's components. Cinnamon leaf extracts collected from Belok Sidan Village, Petang District and Badung Regency, Bali, contained phytochemical compounds, such as, steroid, flavonoid, phenolic, and tannin groups (Darmadi *et al*., 2015). In a more recent study, cinnamon leaf extracts collected from the Bedugul area, Baturiti District, Tabanan, Bali revealed that it contained similar phytochemical compounds. Additional compounds like alkaloids and saponins were also existing in the cinnamon leaf extract of Bedugul samples (Darmadi *et al.*, 2021). The phytochemical content of a plant appeared to attain influences from both biotic and abiotic factors (Darmadi *et al.,* 2015, 2021).

Biotic factors include plants' origin and genetic makeup, and plants from distinct species will produce different phytochemical compounds. The phytochemical content of diverse species belongs to the genus *Pennisetum,* containing varied phytochemical compounds. *Pennisetum pedicellatum* restrains steroids in its leaves and a higher level of alkaloids in its roots. Species *P. pulpureum* contains tannins, alkaloids, phenols, and flavonoids with percentages of 67.9%, 26.21%, 1.16%, and 4.74%, respectively (Ojo *et al*., 2022). However, *P. glaucum* contains tannins, terpenoids, flavonoids, phenols, and glycosides (Ndiku and Ngule, 2015).

Abiotic factors, such as soil and environmental conditions, also manage the variations found in the phytochemical content of a plant. Similarly, soil nutrients result in different essential oil compositions of *Curcuma mangga* samples collected from various locations in Yogyakarta, Indonesia. Essential oils of plant rhizomes planted in the low altitude of Sedayu District and from Beringharjo Market contained higher variations of compounds than those samples collected from Girimulyo, located at an elevated altitude (Astuti *et al.*, 2014). Soil-varied fertility and water availability also cause the diversity in growth-related traits of the Javanese sengon plant (Susanto and Baskorowati, 2018).

The genetic diversity of *Caragana microphylla* (Leguminosae) plants collected from three different sandy areas in North China revealed that *C. microphylla* has a higher variability in regions with diverse temperatures and humidity (Huang *et al.*, 2016). A genetic variation among the plant populations often

occurs due to variations in the climate and its regulation (Keller *et al.,* 2011). Several studies also showed the real impact of climate change on the diversity and genetic structure of the species composition (Fridley *et al.,* 2011; Wu *et al.,* 2012). Disparities in geography, natural selection, and landscape ecology also influence the genetic variation among the species and populations (Nevo, 1998). Based on the above rationale, the current research aimed to know whether biotic and abiotic factors affect the phytochemical content of the cinnamon leaf extract.

MATERIALS AND METHODS

Experimental site and procedure

Fresh cinnamon leaf samples came from two chosen locations: a) Belok Sidan Village, District Petang, Badung Regency, Bali Province, and b) Bedugul Village, District Baturiti, Tabanan Regency, Bali Province, Indonesia. In each location, the collected cinnamon leaf samples were from adjacent plants about 100 m apart, with four replications (Figure 1). Sampling commenced in August 2022.

DNA PCR analysis

DNA extraction

Following the Geneaid method, it is a modification of Vogelstein and Gillespie (1979). Cinnamon leaves, crushed with a pestle and mortar, had 50–100 mg of it transferred into a 1.5 ml tube, then mixed with 400 µl GP1-Buffer and 5 µm A-RNase before homogenizing with a vortex. Then, the tube's incubation continued in a water bath at 60 °C for 10 minutes. Afterward, the cylinder received 100 µl GP2- Buffer (earlier heated to 60 °C), then vortexed and incubated on ice for three minutes. The cinnamon leaf mixture and its solution continued to transfer into a 2-ml filter column and acquired centrifuging at 1000 rpm for one minute. Removing the filter caused the supernatant to relocate into a new 1.5 ml tube, with the GP3-Buffer added to as much as 1.5× the supernatant volume and vortexed for five seconds. Then, placing 700 µl of the mixture in the GD column-2 ml received further centrifugation at 14,000–16,000 rpm for two minutes.

Figure 1. Research locations on the map of Bali Island, Indonesia.

The precipitate's removal had the GD column placed back into the tube. Then, putting a total of 400 µl W1- Buffer into the GD column, continued its centrifugation at 14,000–16,000 rpm for 30 seconds. The sediment's removal led the GD column to return to the tube. The washing process consisted of 600 µl Wash-Buffer, then 400 µl ethanol. After washing the ethanol, the GD column's drying followed by centrifuging at 14,000–16,000 rpm for 30 seconds. Then, the dry GD column's transfer to a new tube continued. The addition of 100 µl of heated Elution-Buffer ensued to the cylinder, leaving it for five minutes, and again, centrifuged at 14,000–16,000 rpm for 30 seconds. The obtained substance is the resulting DNA extract stored in the refrigerator.

The Epicentre method follows the protocol of the Epicentre kit, which is a modification of Steiner *et al.* (1995). Cutting cinnamon plant leaves, measuring 3 mm × 5 mm, proceeded into the tube, with 100 µl of Quick Extract Plant DNA Extraction Solution added to the vessel to immerse the leaf pieces in the solution. Then, the sample gained heating in a water bath at 65 °C for six minutes and continued heating at 98 °C for two minutes. Removing leaf pieces from the tube left the solution, becoming the result of DNA extraction to be used directly for the PCR reaction.

The extracted DNA's fractionation continued electrophoretically in 1% agarose gel with TBE buffer (Tris-EDTA). Electrophoresis processing ensued with an electric current of 80 volts for 60 minutes. Then, the electrophoretic gel's immersion for 10 minutes in TBE buffer followed, which comprised 5 ul ethidium bromide at a concentration of 1 µg ml-1. The soaked gel received visualization using a UV-transilluminator.

DNA amplification by PCR

Two universal barcoding markers' use identified the cinnamon species in the two locations, *rbcL* and *matK* genes. The *rbcL* gene amplified utilized rbcLa-F forward-(ATGTCACCACAAACAGAGACTAAAGC) and rbcL724R reverse (TCGCATGTACC TGCAGTAGC) primers (Kress and Erickson, 2007; Fay *et al.,* 1997). The *matK* gene amplification used MatK_390f forward-(CGATCTATTCATT CAATATTTC) and MatK_1326R reverse-(TCTAGCACACGAAAGTCGAAGT) primers (Cuenoud *et al.*, 2002).

The reaction took place using a personal Takara PCR thermal cycler (Takara Bio, Otsu, Japan) with Ex Tag (Takara Bio, Otsu, Japan) under the following conditions: pre-denaturation at 94 °C (4 min), followed by 35 cycles of denaturation 94 °C (35 s), annealing at 52 °C (55 s), elongation 72 °C (2 min), and post-elongation at 72 °C for 10 min (Nishizawa *et al.*, 2010).

Computer analysis of DNA sequences

Nucleotide sequences' determination employed the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), according to the tool's instructions and with a PE Applied Biosystems Automated DNA Sequencer (model 3130xl, Applied Biosystems). Double-helical DNA sequences' assemblage incurred analysis using Genetyx (version 11.0) and Genetyx-ATSQ (version 4.0) software (Genetyx, Tokyo, Japan), respectively, and compared with the equivalent DNA sequences retrieved from the DDBJ/EMBL/GenBank via NCBI BLAST program (Thompson *et al.,* 1997).

Phylogeny analysis used the MEGA 4.0 program (Kumar *et al.,* 2001) and the Neighbor-Joining (NJ) method with bootstrap 1000x, with the following steps: a) Identifying the similarity between sequences, sequence data stored in a notepad in FASTA format underwent analysis using the Blast-WU facility available online at www.ebi.ac.uk/Clustalw. Based on similarity analysis, if observations reveal the degree of similarity in the base sequence of the 18S rRNA encoding gene is less than 97%, it can receive classification as a new species (Pangastuti, 2006); b) Making a phylogeny tree with the MEGA program with the processed data using the ClustalW facility, to serve as basic data for creating phylogenetic trees using the MEGA data facility.

Soil sampling

Soil collection around the locations of the cinnamon plants transpired using a bamboo measuring 30 cm long. Sticking the bamboo to a depth of 30 cm, the bamboo's removal from the ground had the soil in the stick poured into the container. The soil retrieval continued three times. The collected soil samples' analysis for soil classification continued at the Laboratory of the Faculty of Agriculture, University of Udayana, Indonesia. Soil classification includes pH, DHL, organic C content, total N, available P and K (ppm), water content consisting of DA (%) and FC (%), and soil texture.

Climate data

Climate data collection included temperature, humidity, and wind speed from the Center for Meteorology, Climatology, and Geophysics Region III Denpasar, Bali, Indonesia. The coordinate data used the 'Coordinate Map' software at N45 and E25, with the altitude taken based on the 'Accurate altimeter' application software, two applications downloaded from the Samsung Galaxy A71.

RESULTS AND DISCUSSION

Cinnamon identification based on PCR-DNA analysis

The analysis for cinnamon identification using plant leaves commenced with two distinct marker genes, namely, *matK* (maturase-K gene) and *rbcL* (ribulose-bisphosphate carboxylase) genes. Based on the results, the cinnamon plants of the two locations, i.e., Petang and Bedugul, probably belonged to the same species. The analysis using the *rbcL* gene indicated that the cinnamon species collected from both habitats and locations was *Cinnamomum tamala* or *C. osmophloeum* (Figures 2 and 3).

Cinnamon tree phylogeny using a maximum likelihood method with MEGA 4.0 software showed the kinship between species in the genus *Cinnamomum* and other species in the family Lauraceae (Fitmawati *et al*., 2022). Cinnamon samples from Belok Sidan Village, District Petang, Tabanan, Bali (A2), and Bedugul Village, District Baturiti, Tabanan, Bali, Indonesia (B2), form a separate clade with *C. tamala,* and with *C. osmophloeum* (Figure 3).

The maturase-K (*matK*) gene and the ribulose-bisphosphate carboxylase (*rbcL*) gene are universal marker genes commonly used to identify the species of crop plants. It may be due to the existence of these sequences in almost all plants; however, it is specific enough to distinguish the plants. In the presented study, using both genes helped identify the types of cinnamon plants found in the Petang area (sample label 'A') and in the Bedugul area (sample label 'B'). The PCR results using *matK* and *rbcL* primers showed specific amplification for all tested samples, as indicated by a single band that appeared on the electrophoresis gel. It confirmed that the sequences of the two genes on the cinnamon plant genome have specificity, making them useful in the sequencing reactions.

Alignment of the 18s rDNA of the eight samples (four each from Petang and Bedugul), using the *matK* gene, indicated that all plant samples probably belonged to the same plant species. The alignment of the *rbcL* gene sequences between sample A2 (Petang) and sample B2 (Bedugul) also provided the same pattern, where both sequences were 100% identical. From these results, it is inherent that the cinnamon plants collected from both locations (Petang and Bedugul) conformed to the same species. The use of the *matK* marker gene and the *rbcl* gene to identify a species also has results from other researchers in the past (Swetha *et al*., 2016; Dukenov *et al*., 2023). The study reported that the marker genes *rbcL*, *matK*, *psbA-trnH*, and Internal Transcribed Spacer (ITS) could identify *Myristica fragrans,* which differed from *M. malabarica*. The potential of psbA-trnH showed the best barcode over other loci in *M. fragrans* authentication. Bansal *et al*. (2018) reported that ITS2, *rbcL-a*, *mat-K*, and *psbA-trnH* marker genes could distinguish the *Bunium persicum* and *Cuminum cyminum*. The *B. persicum* spice, commonly known as Kala

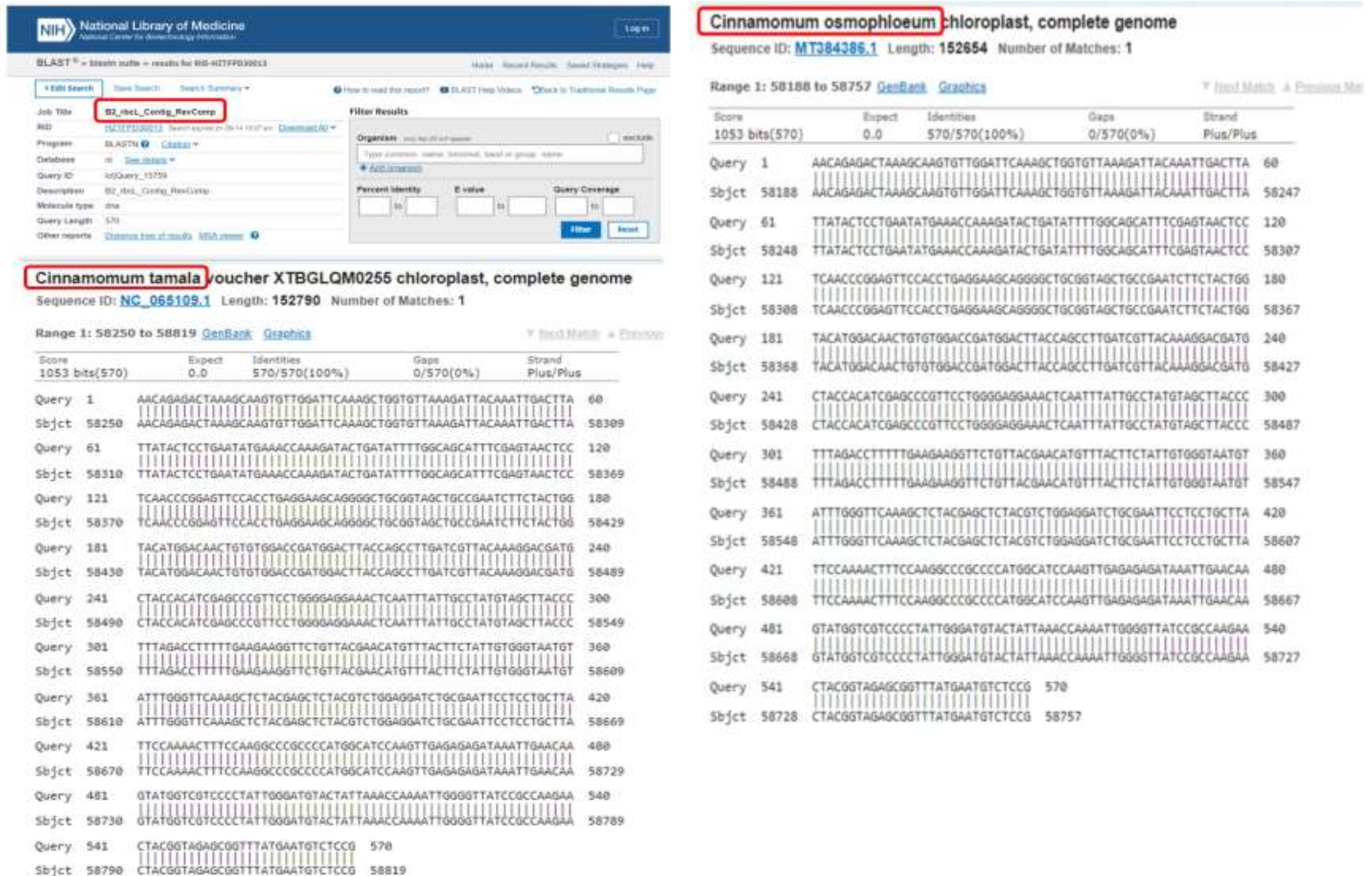

Figure 2. Blast results of the rbcl gene sequence of cinnamon plants collected from Bedugul Village sample, Baturiti District, Tabanan, Bali, Indonesia (B2) showing 100% identical to the rbcl gene of *Cinnamomum tamala* and *C. osmophloeum.*

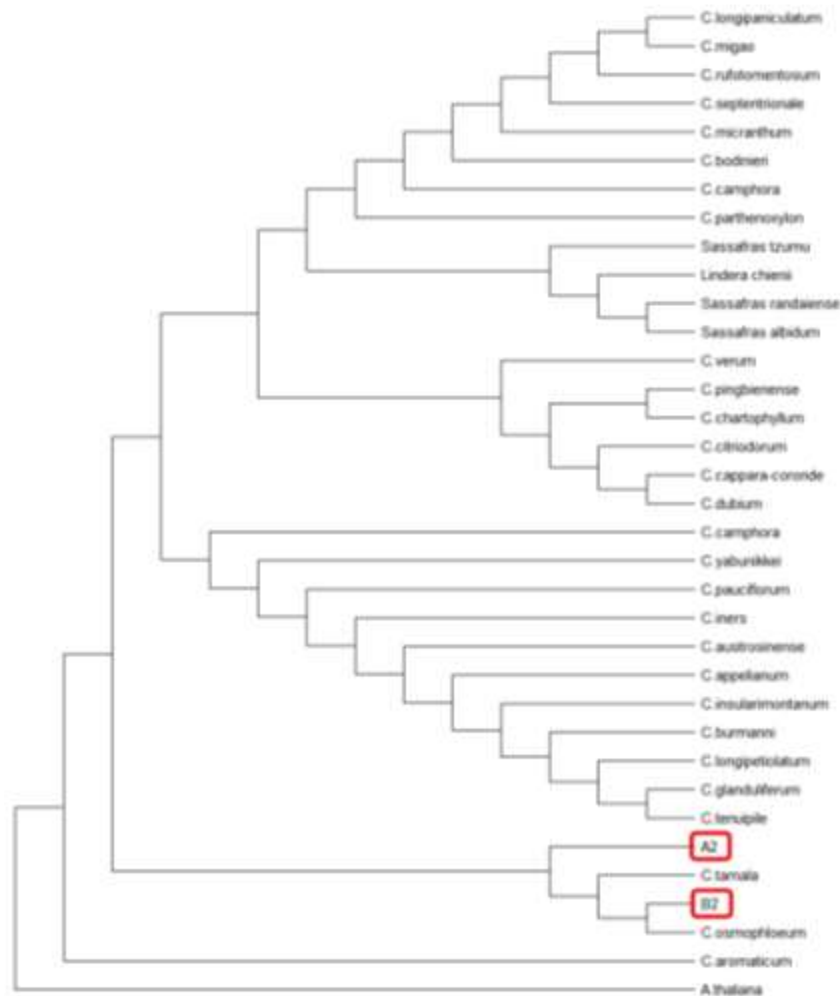

Figure 3. Phylogenetic tree construction using the Maximum likelihood method with MEGA 4.0 software-showing kinship between species in the genus *Cinnamomum* and with other species in the family Lauraceae, based on the rbcl gene sequence. Cinnamon Petang (A2) and Bedugul (B2) samples form a separate clade with *C. tamala* and *C. osmophloeum.*

zeera, is a spice that has very high value for medicinal purposes. This spice's adulteration often occurred with *Cuminum cyminum* or Safed zeera, a closely related species. Among the tested barcodes, loci, psbA-trnH has proven to be the best authenticating barcode.

A phylogenetic tree can be advantageous to ensure whether or not a plant is closely related to other plants. Phylogenic tree construction using the maximum likelihood method with MEGA11 software showed a relationship among species in the genus of *Cinnamomum* and other species in the family of Lauraceae. Based on the *rbcl* gene sequence, the cinnamon samples of Belok Sidan Village, Petang District, Badung (A2) and Bedugul Village, Baturiti District, Tabanan (B2), form a separate clade with *Cinnamomum tamala* and *C. osmophloeum*. It authenticated that the cinnamon plants found in Belok Sidan, Petang, Badung, and Bedugul Village, Baturiti District, Tabanan Bali, Indonesia, were closely related to the species of *C. tamala* and *C. osmophloeum*. Several studies on kinship examined the genus *Myrcia* from the Myrtaceae family, and based on the phylogenetic tree, the

Myrceugenia group was a sibling of the Myrcia and Plinia groups (Amorim *et al*., 2019). A Korean endemic plant, *Fraxinus chiisanensis*, and Fraxinus phylogeny based on two nuclear DNA (nrITS and phantastica) and two chloroplast DNA (psbA-trnH and rpl32-trnL) could identify *F. chiisanensis* as a genetically distinct unit from its sister group, *Fraxinus platypoda* from Japan (Kim *et al.*, 2021).

Soil analysis

The analysis of the soil samples collected from both locations: a) Belok Sidan Village, Petang District, Badung Regency (A1-A4), and b) Bedugul, Baturiti District, Tabanan Regency (B1-B4) are available in Table 1. Both soil samples had an equal pH of 6.8 and a neutral category. Also, both specimens had a DHL value of 0.4 mm hos/cm, categorized as very low. The organic C of soil samples collected from Bilok Sidan and Bedugul areas were 2.34 (medium category) and 3.15 (high classification), respectively. The N, P, and K contents of soil samples collected from the Bilok Sidan were 0.27% (medium), 8.01 ppm (lowest), and 190.1 ppm (moderate category), respectively. However, the sample collected from the Bedugul area had N, P, and K values of 0.65% (high), 6.27 ppm (lowest), and 224.03 ppm (high category), respectively.

The water content of Bilok Sidan soil samples was 4.46% DA and 38.2% FC, with a soil texture consisting of 62.15% sand, 19.34% silt, and 18.6% clay, including sandy loam soil (Table 1). The Bedugul soil samples had a water content of 7.4% DA and 40.74% FC, with a soil quality of 65.17% sand, 18.32% silt, and 16.51% clay, including sandy loam soil consistency. The C, N, and K contents of the soil sample collected from the Bedugul Village (B1-B4) were higher than those of Belok Sidan Village (A1-A4). The differences in soil samples could result in different phenotypes and phytochemical content of vegetation.

The soil conditions significantly determined the vegetation’s yield and secondary metabolite contents. Plants growing on soils with higher stress conditions tend to contain higher secondary metabolites than those with less stress conditions. The plant’s secondary metabolites greatly sustain influences from variations in environmental factors (Li *et al*., 2020). Secondary metabolite contents of crop plants have several ecological factors influencing these, such as, soil fertility and water content, salt content, light, and temperature (Yang *et al.,* 2018). Drought stress also significantly affects the physiological processes and the formation of plant secondary metabolites. Drought stress factors enhance the quality of secondary metabolites’ development, such as rutin, quercetin, and Betulinic acid in *Hypericum brasiliense* plants and artemisinin in Artemisia plants (Verma and Shukla, 2015).

Table 1. Soil analysis of the two sampling locations (Belok Sidan Village, Petang Badung District and Bedugul Village, Baturiti District, Tabanan), Bali, Indonesia.

No.	Sample	Ph (1;25)	DHL (mm hos/cm)	C organic (%)	N Total (%)	P available (ppm)	K available (ppm)	Water content		Texture		
		H_2O						DA (%)	FC (%)	Sand (%)	Dust (%)	Clay (%)
1	A1-A4	6.8 (N)	0.4 (VL)	2.34 (M)	0.27 (M)	8.01 (VL)	190.1 (M)	4.46	38.2	62.15 Sandy loam	19.34	18.6
2	B1-B4	6.8 (N)	0.4 (VL)	3.15 (H)	0.65 (H)	6.27 (VL)	224.03 (H)	7.4	40.74	65.17 Sandy loam	18.32	16.51

Information: N = Neutral, VL = Very Low, M = Medium, H = High, DA = Dry Air, FC = Field Capacity

Soil conditions, such as salt stress, also affect secondary metabolites in onion (*Allium cepa*), and sodium carbonate and sodium bicarbonate application at various concentrations (0, 10, and 100 m/M) to *A. cepa* plants proved to increase the concentrations of superoxide dismutase, glutathione peroxidase, and catalase (Sivasamy *et al.*, 2022). Salt stress affects secondary metabolites in plants of the Lamiaceae tribe. Moderate soil salinity impacts secondary metabolites, namely, terpenoid and phenolic components in plants belonging to the Lamiaceae family. Plants belonging to the Lamiaceae tribe are medicinal and aromatic plants that produce secondary metabolites for the pharmaceutical, cosmetic, and food industries (Assaf *et al.,* 2021). The effects of environmental factors on the phenotypic changes in 17 cinnamon plant samples in five districts, i.e., Kerinci, Full River, Merangi, Bungo, and Soralangun, Jambi province, Indonesia revealed that based on similarity coefficient (58.23), the cinnamon samples divide into two groups, i.e., group 1 consisted of 16 accessions, and group 2 consisted of one accession (Lizawati *et al*., 2018).

Climate analysis

The Belok Sidan Petang Village, Badung, had an average daytime temperature of 23 °C, 83% humidity, wind speed at 13 km/h, altitude of 947–967 masl, and average annual rainfall per year of 2,135 mm. However, the Bedugul Village, Baturiti District, had an average daytime temperature of 22 °C), 65% humidity, wind speed of 14 km/h, altitude of 1280-1286 masl, and average rainfall per year at 2,509 mm. Environmental conditions of both locations used for cinnamon sample collections are available in the Meteorological and Geophysical data, August 4, 2022 (Table 2).

On average, both sampling locations' climatic conditions (temperature, humidity, wind speed, coordinates, and altitude) slightly differed, which certainly affected the secondary metabolites' yield in cinnamon plant leaves. An area's temperature has reports of affecting the vegetation's secondary metabolite content. The presented results may have varying temperatures influencing these from both sample collection locations. Duration of light exposure also has claims of influences from air temperature and, hence, affecting plants' secondary metabolite content. The sunlight, especially UV-B, also impacts the photosynthetic process and the secondary metabolite content of plants. UV-B exposure for 13 days on *Nymphoides humboldtiana* plants apparently increased their photosynthetic rate and contents of secondary metabolites (particularly flavonoids) (Nocchia *et al.*, 2020).

Table 2. Data on temperature, humidity, wind speed, and altitude at the two sampling locations.

No.	Location	Temperature (°C)	Humidity (%)	Wind speed (km/h)	Coordinates	Altitude (m)
1	A1 (Belok Sidan, Petang)	23	83	13	S8°17′26.4264″E 115°14′28.0536″	953
2	A2 (Belok Sidan, Petang)	23	83	13	S8°17′11.7528″E 115°14.45.6216″	947
3	A3 (Belok Sidan, Petang)	23	83	13	S8°17′27.708″E 115°14′28.2912″	957
4	A4 (Belok Sidan, Petang)	23	83	13	S8°17′28.824″E 115°09′48.2292″	967
5	B1 (Bedugul, Baturiti)	22	65	14	S8°16′58.2528″E 115°09′48.2292″	1280
6	B2 (Bedugul, Baturiti)	22	65	14	S8°16′58.764″E 115°09′48.1464″	1283
7	B3 (Bedugul, Baturiti)	22	65	14	S8°16′59.754″E 115°09′45.9972″	1281
8	B4 (Bedugul, Baturiti)	22	65	14	S8°17′00.222″E 115°09′47.2248″	1286

Abiotic factors, such as drought, temperature, and sunlight, enhanced the content of secondary metabolites in medicinal plants, especially those used in pharmaceutical and aromatic industries (Thakur *et al.*, 2019). Similarly, the different climatic conditions can affect the secondary metabolites of a plant. Kinnow mandarin plants grown under different climates revealed that the Kinnow mandarin fruit grown in subtropical climates produced a superior content of polyphenols and limonoids than those grown in other climates (Saini *et al*., 2019). The temperature increases resulted in variations in the plant secondary metabolites in Australian and Canadian wheat plants, and the total phenolic content enhanced with higher temperatures (Shamloo *et al.*, 2017).

CONCLUSIONS

Based on the results, the cinnamon plants found in two locations (Belok Sidan Petang Village, Badung, and Bedugul Baturiti Tabanan Village), Bali, Indonesia, belonged to the same species, namely, *Cinnamomum tamala* or *C. osmophloeum*. The phytochemical content of cinnamon leaf samples collected from those two habitats slightly differed due to varied soil and climatic conditions.

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