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GENETIC DIFFERENCES BETWEEN *DENDROBIUM CHRYSOTOXUM* NATIVE TO NORTHEASTERN AND NORTHERN REGIONS OF THAILAND BASED ON *GALANTHUS NIVALIS* AGGLUTININ-RELATED LECTINS AND INTERNAL TRANSCRIBED SPACER REGIONS OF RIBOSOMAL DNA

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Abstract

Dendrobium chrysotoxum Lindl., native to the Northeastern region and that native to the Northern region of Thailand have some different morphological characters. Their genetic differences were then determined at molecular level. *Galanthus nivalis* agglutinin (GNA)-related lectins were purified from the pseudobulbs using mannan-agarose column chromatography. The molecular masses of polypeptide subunit and native form, and pI of GNA-related lectin were 13.9 kDa, 54.1 kDa, and 5.16 for *D. chrysotoxum* (Northeast) while they were 13.7 kDa, 48.2 kDa, and 4.90 for *D. chrysotoxum* (North), respectively. The sequence tags from liquid chromatography-tandem mass spectrometry (LC-MS/MS) of both lectins were identical to parts of a lectin precursor from *D. findleyanum*. The lectins exhibited divergent abilities to agglutinate erythrocytes and were named *D. chrysotoxum* agglutinin-Northeast (DCA-NE), and *D. chrysotoxum* agglutinin-North (DCA-N). For binding activities, the activities of DCA-NE and DCA-N decreased after treatment with 2-mercaptoethanol; they also showed different specificities towards D-mannose. The lectins showed variation at nucleotide and deduced amino acid sequences. From internal transcribed spacer (ITS) sequences, the genetic distance between the two orchids had greater value than those among many *Dendrobium* species. The molecular data revealed that *D. chrysotoxum* native to the different geographical regions of Thailand had a large genetic difference; and should be placed to be as two species or at least two varieties of the genus.

Key words: Genetic differences, *Dendrobium chrysotoxum*, GNA-related lectins, ITS regions

Introduction

At the present, biological macromolecules are gaining an increasingly important role in systematic and evolutionary studies (Onarici & Sumer, 2003; Jamil *et al*., 2014), as well as in application for genetic markers (Kumar *et al*., 2009; Sultan *et al*., 2013). Protein and DNA analyses are widely used to generate systematic data. For protein analysis, isozymes and allozymes were mainly utilized for the systematic purpose and as the markers. However, more useful data are now received from DNA analysis. Several procedures have been used to obtain DNA data, for example, DNA sequencing, random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP) (Bremer, 1988; Onarici & Sumer, 2003; Simpson, 2010; Akbar *et al*., 2011). In plant, the major sources of DNA sequence data consist of nuclear DNA, chloroplast DNA, and mitochondrial DNA. Internal transcribed spacer (ITS) region of rDNA is one of the very useful types of nuclear DNA sequences (Simpson, 2010). This region has proven to be a useful source of characters for phylogenetic studies in closely related species (Baldwin *et al*., 1995; Simpson, 2010).

Dendrobium is one of the largest genera in Orchidaceae and contains about 1,250 species which distribute widely in Asia to Australia and Pacific region (Mabberley, 2008). Morphological character alone which is the traditional tool for identification of *Dendrobium* species is still ambiguous because the orchids have great diversification of the characters (Takamiya *et al*., 2011). Therefore, the molecular data are needed for systematic studies of *Dendrobium* species. Internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA) were used for re-identification of *Dendrobium* species; the result indicated that *Dendrobium* (*D*.) *somai* and *D. furcaptopedicellatum*, and *D. moulmeinense* should be placed outside genus *Dendrobium* (Tsai *et al*., 2004; Yuan *et al*., 2009). The ITS regions were also used to distinguish *Dendrobium* species which were used in traditional Chinese medicine from adulterants (Xu *et al*., 2006; Takamiya *et al*., 2011; Wu *et al*., 2012).

In addition to the great diversity of the orchids, *Dendrobium* substances have been used in traditional Chinese medicine in China and Asian countries (Bulpitt *et al*., 2007; Xue *et al*., 2010). Lectins are one of bioactive constituents found in *Dendrobium* species (Ng *et al*., 2012). *D. officinale* agglutinin2 (DOA2) and *D. findleyanum* agglutinin (DFA), which are *Galanthus nivalis* agglutinin (GNA)-related lectins (previously called monocot mannose-binding lectins), with their biochemical properties were reported (Chen *et al*., 2005a; Sudmoon *et al*., 2008; Sattayasai *et al*., 2009). We are interested in the study of GNA-related lectin from *D. chrysotoxum* Lindl., because the stem of this orchid is used in both traditional Chinese and folk medicine (Yang *et al*., 2004). *D. chrysotoxum* found in Thailand has two

morphological characters; *D. chrysotoxum* from the Northeastern region has very similar morphology to the species described by Cullen (1992) while the plant from the Northern region has very similar morphology to the species described by Schettler (2006).

In this study, we used both morphological characters and molecular data to study the genetic differences between *D. chrysotoxum* native to the two regions of Thailand. The molecular data included biochemical properties and deduced amino acid sequences of GNA-related lectins, and sequence of ITS regions. The results showed that there were many differences between *D. chrysotoxum* from both regions and there is a possibility of segregation of the two ecotypes into at least two different varieties.

Materials and Methods

Plant material: *Dendrobium chrysotoxum* plants were collected from the Northeastern and Northern regions of Thailand (Fig. 1). The mature pseudobulbs were used for crude protein extraction and total RNA extraction while the fresh leaves were used for total DNA extraction. Voucher herbarium specimen of *D. chrysotoxum* from the Northeastern region (KKU 2152) and that from the Northern region (KKU 2153) have been deposited at the herbarium of Khon Kaen University, Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand.

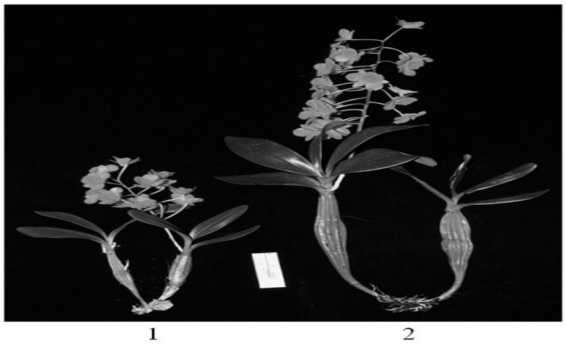

Fig. 1. Pseudobulbs with leaves and flowers of *D. chrysotoxum*; 1 = The plant collected from the Northeastern region of Thailand, 2 = The plant collected from the Northern region of Thailand.

Crude protein extraction: Crude protein extraction was performed following the protocol reported by Sudmoon *et al*., (2008) with some modifications. A 0.4 g of plant tissue was ground in 0.5 ml of extraction buffer (100 mM Tris-HCl, 20 mM EDTA, pH 8.0, containing 5 mM 4-aminobenzamidine dihydrochloride, and 1 mM phenylmethylsulfonyl fluoride) with mortar and pestle at 4°C. The homogenate was centrifuged at 18,000×*g* for 25 minutes at 4°C. The supernatant was collected as crude protein extract.

Affinity chromatography: Mannose-binding protein was purified from the crude protein extract using mannan-agarose (Sigma; Sigma-Aldrich Chemie GmbH, Germany) column chromatography following the procedure described by Sudmoon *et al*., (2008) with slight modification. After the eluted protein was adjusted to pH 7 with 1 M Tris base, it was then washed with a solution of 10 mM Tris-HCl, 2 mM EDTA, pH 8.0 by means of molecular filtration (Amicon® Ultra-4; Millipore, Ireland). The purity of the protein was determined by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a continuous gradient of 8-20% acrylamide.

SDS-PAGE: The SDS-PAGE was performed on a Mini-Protean II Dual Slab Cell (Bio-Rad Laboratories, USA) using the discontinuous buffer system as described in Sudmoon *et al*., (2008) except that the gel was shaken for 15 minutes in a fixing solution (50% methanol and 12% acetic acid) prior to staining.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and database search: The protein bands in the gradient SDS-PAGE gel with molecular masses of 13.7 kDa, 13.9 kDa and 24 kDa of *D. chrysotoxum* were excised from the gel, reduced with 10 mM DTT and alkylated with 100 mM Iodoacetamide. After digestion with trypsin for overnight, peptides were extracted and then separated by Ultimate 3000 LC system (Dionex, Germany). Peptides were subsequently analyzed by ESI-MS/MS using an ion trap MS (HCT ultra PTM Discovery System; Bruker Daltonik, Germany). All collected LC-MS/MS data were used as the input for MASCOT MS/MS Ions Search of the National Center for Biotechnology Information nonredundant (NCBI nr) database (www.matrixscience.com).

Hemagglutination assay: Hemagglutination activity of the purified lectins was determined with trypsinized chicken erythrocytes according to the procedures described by Ola *et al*., (2007) with some modifications. Fresh blood of chicken was collected in 3-ml tube containing EDTA. It was centrifuged at 430×*g* for 10 minutes at 10°C, and the supernatant was then removed. After washing 4 times with phosphate buffered saline 1 (PBS 1) (50 mM NaH_2PO_4, pH 7.4, containing 150 mM NaCl), 5% chicken erythrocyte suspension in PBS 1 containing 1 mg/ml trypsin (Sigma Chemical, USA) was prepared and mixed by inversion and then incubated for 2 hours at 37°C. The tube was inverted every 15 minutes. The suspension was centrifuged, washed for 4 times, made into 40% erythrocyte suspension with PBS 1. The 40% erythrocyte suspension was diluted into 4% erythrocyte suspension with PBS 2 (137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4). Two-fold serial dilution of 50 µl purified lectin in PBS 2 was incubated with 50 µl of 4% chicken erythrocyte suspension in U-shaped microtiter plate. Visible agglutination was observed after incubation of the plate for 30 minutes at room temperature.

Native-PAGE and ferguson plot: Native-polyacrylamide gel electrophoresis (native-PAGE) was performed as previously described (Sudmoon *et al*., 2008) and Ferguson plot was done to determine molecular masses of the purified proteins (Kong *et al*., 1991).

Isoelectric focusing (IEF): IEF was performed as described by Sudmoon *et al*., (2008) except that 5.5% acrylamide was used for the slap gel and 4 washes of the destaining solution (40% methanol and 10% acetic acid) were done before staining the gel with Coomassie brilliant blue R-250.

Solid-phase method: The mannose-rich glycoprotein horseradish peroxidase (HRP) (Heth & Bernstein, 1991) was used to test the binding activity of the purified lectins in a microtiter plate according to the procedures described by Sudmoon *et al*., (2008) except that 3 µg of DCA-NE or 5 µg of DCA-N was incubated in each well of F96 Maxisorp Immuno plate (Nunc, USA) for 1.5 hours at 37°C. In order to determine the effect of 2-mercaptoethanol on binding activity of the lectins, 0.28 or 0.70 M 2-mercaptoethanol was present in the lectin solution. For binding competition, D-mannose (10, 30, 50, 70 or 90 mM), D-galactose, D-glucose, D-xylose or D-ribose (30, 90, 150 or 180 mM) was present in the HRP solution. The percentage of binding activity was calculated against the positive control (without 2-mercaptoethanol, or without competition with sugar) which was designed as 100% activity.

Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) of lectin genes: Total RNA was extracted from the pseudobulbs according to the procedures described by Bekesiova *et al*., (1999). Synthesis of cDNA was done by using ImProm-II™ reverse transcriptase (Promega, USA) following to the company's instruction. The RT reaction was performed in DNA Engine® PTC-200 Peltier Thermal Cycler (MJ research, USA).

For PCR, the forward primer was 5′-CACCCAAGTAGAGTACAACCAAGAAC-3′, and the reverse primer was 5′-CAACCAACTCGTACGTACA CAAACC-3′ (Invitrogen, USA). These primers were designed from upstream sequence and downstream sequence of the open reading frame of *D. officinale* agglutinin (DOA) cDNA (Chen *et al*., 2005b). The PCR mixture (25µl) contained 1X GoTaq® Green Master Mix (Promega), 0.5 µM each primer, and cDNA prepared from 80 ng of total RNA template. The PCR reaction was performed in the same machine under the following conditions: initial denaturation for 3 minutes at 94°C, followed by 35 cycles of amplification (denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C, extension for 2 minutes at 72°C) and final extension for 7 minutes at 72°C. The PCR products were separated and detected using 1.5% agarose gel electrophoresis in 0.5X Tris-acetate-EDTA (TAE) buffer and stained by 0.5 µg/ml of ethidium bromide.

DNA sequencing and data analysis of the lectin genes: The PCR products were purified by using Wizard® SV Gel and PCR Clean-Up System (Promega), inserted into pGEM®-T Easy Vector and then transferred into *Escherichia coli* JM109 (Promega). PCR reaction with M13 forward primer (5'-GTAAAACGACGGCCAGT-3') and M13 reverse primer (5'-GGAAACAGCTATGACCATG-3') was done by using DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, USA) in PCR Sprint Thermal Cycler (Thermo Fisher Scientific, USA). The thermal cycle was 30 cycles of amplification (denaturation at 90°C for 20 seconds, annealing at 50°C for 15 seconds, extension at 60°C for 1 minute). The sequencing was performed for both 5' and 3' directions in MegaBACE 1000 (GE Healthcare).

By using BioEdit (version 7.0.8), the nucleotide sequence obtained from the reverse primer was reverse-complemented and then aligned with the sequence obtained from the forward primer to receive the completed sequence data for each sample. Each completed sequence was then analyzed by using Biology Workbench 3.2 to get possible six open reading frames (ORF). The amino acid sequence translated from the longest ORF was used to predict a signal peptide by using SignalP 3.0 server. It was also used for searching of similar sequences in the website of NCBI using Protein BLAST and then compared with the most similar sequences by using ClustalW2. Theoretical molecular masses of the lectin precursors were computed by using ExPASy Compute pI/Mw tool.

Total DNA extraction and PCR amplification of the ITS regions: Total DNA was extracted from fresh leaves of *D. chrysotoxum* native to the Northern region by using CTAB method (Porebski *et al*., 1997). The extracted DNA was qualified using 0.8% agarose gel electrophoresis in 0.5X TAE. The approximate DNA concentration was quantified using a Jasco V-530 UV/Vis spectrophotometer (Jasco, Japan).

IT1: 5′–TCGTAACAAGGTTTCCGTAGGT–3′ and IT2: 5′–GTAAGTTTCTTCTCCTCCGCT–3′ (Invitrogen) were the forward and reverse primers, respectively (Tsai *et al*., 2004). The PCR mixture (20 µl) contained 1X GoTag® Green Master Mix (Promega), 0.25 µM each primer, 40 ng DNA template, and additional 0.5 mM $MgCl_2$. Amplification was performed in GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems, USA). Temperature profile was the same as those used for PCR portion of the RT-PCR previously described. The PCR products were separated on a 1.2% agarose gel electrophoresis in 0.5X TAE buffer, and stained by 0.5 µg/ml of ethidium bromide.

DNA sequencing and data analysis of ITS regions: The fragment, at approximately 750 bp, of the amplified rDNA region was purified from the agarose gel using Perfectprep Gel Cleanup Kit (Eppendorf AG, Germany) according to the manufacturer's instructions. The purified fragment was sequenced using the same primers used for the PCR. The fragment was sequenced for both directions in MegaBACE 1000 (GE Healthcare) as described above. Genetic relationships of *D. chrysotoxum* (Northeast), *D. chrysotoxum* (North) and other *Dendrobium* species based on ITS sequences were determined by using MEGA version 4.0 (Tamura *et al*., 2007). *Bulbophylum* (*B*.) *blepharistes* was used as the outgroup species. The genetic distance matrix was calculated from sequence alignment by the two-parameter model (Kimura, 1980), and then was used to construct a phylogenetic tree by using Neighbor-joining method (Saitou & Nei, 1987) with bootstrap tests of 1,000 replicates (Felsenstein, 1985).

Results

Morphological characters of *D. chrysotoxum* collected from the Northeastern and Northern regions of Thailand: *Dendrobium chrysotoxum* collected from both areas have common morphological characters as follows. Plant epiphytic. *Stems* sympodial, pseudobulb, fusiform. *Leaves* elliptic, entire, retuse at apex, amplexicaul, distichous. *Inflorescene* lateral. *Flowers* spiral. *Dorsal sepal* and *lateral sepals* oblong, entire. *Petals* obovate. *Lip* hairy at upper side. However, they have different characteristics as shown in Table 1.

Purification of mannose-binding proteins: The mannose-binding proteins of *D. chrysotoxum* were purified from the crude protein extracts by using mannan-agarose column chromatography. From SDS-PAGE in the presence of 2-mercaptoethanol with heating, the mannose-binding protein of *D. chrysotoxum* (Northeast) showed one major band with molecular mass of 13.9 kDa while those of *D. chrysotoxum* (North) gave two major bands with molecular masses of 13.7 kDa, and 24 kDa (Figs. 2 & 3). The minor bands at the lowest position were assumed to be non-glycosylated form of the monomers. Moreover, in the presence of only 2-mercaptoethanol without heating, mannose-binding protein from *D. chrysotoxum* (Northeast) showed additional band at 58 kDa while that from *D. chrysotoxum* (North) exhibited little smear band (Fig. 3).

Protein identification by LC-MS/MS: The protein bands of mannose-binding proteins at 13.9 kDa for *D. chrysotoxum* (Northeast), 24 kDa and 13.7 kDa for *D. chrysotoxum* (North) were identified by LC-MS/MS and NCBI database search using MASCOT. These bands showed the same amino acid sequence and were identified as parts of a mannose-specific lectin precursor from *D. findleyanum* called *D. findleyanum* agglutinin (DFA) precursor (Table 2).

Hemagglutination activity: The ability to agglutinate animal red blood cells of the lectins was examined. The result showed that 0.576 µg of lectin from *D. chrysotoxum* (Northeast), and 1.152 µg of lectin from *D. chrysotoxum* (North) were the lowest amount that could agglutinate trypsinized chicken erythrocytes. Therefore, the proteins were named *D. chrysotoxum* agglutinin-Northeast (DCA-NE), and *D. chrysotoxum* agglutinin-North (DCA-N).

Native form of the lectins: Relative migrations of standard proteins, DCA-NE and DCA-N on native-PAGE with five different concentrations of acrylamide were used to determine molecular masses of native forms of DCA-NE and DCA-N by using Ferguson plots (Figs. 4 & 5). The results showed that DCA-NE had molecular mass of 54.1 kDa while DCA-N showed molecular mass of 48.2 kDa.

Table 1. Comparative morphological characters of *D. chrysotoxum* collected from the Northeastern and Northern regions of Thailand.

Characteristics	*D. chrysotoxum* (Northeast)	*D. chrysotoxum* (North)
Pseudobulb size	10.0-17.5 x 1.0-2.0 cm.	13.0-28.7 x 1.5-3.7 cm.
Pseudobulb shape	8-9 longitudinal grooves	8-11 longitudinal grooves
Leaf size	10.0-16.5 x 1.5-3.5 cm	9.5-17.5 x 2.5-5.1 cm.
Flower color	yellow	deeply yellow
Maximum number of flowers per inflorescence	15	23
Petal margin	denticulate	entire, sometimes cleft
Lip	orbicular, tomentulose hairy at upper side, erose at margin	ovate, tomentose hairy at upper side, lacerate at margin

Table 2. Identification of protein bands on reducing gradient SDS-PAGE, obtained from the purified mannose-binding proteins of *D. chrysotoxum* native to the Northeast, and that native to the North of Thailand, using LC-MS/MS and database search.

Protein band	Peptides identified by LC-MS/MS	Mr (expt)	Mr (calc)	Protein identification
	***D. chrysotoxum* (Northeast)**			
13.9 kDa	Neaiwask	917.4578	917.4606	Mannose-specific lectin precursor from *D. findleyanum*
	Tdgengnyviilqk	1,562.8024	1,562.7940	
	Dgnlvlyskpifatgtnr	1,964.9827	1,965.0320	
	***D. chrysotoxum* (North)**			
13.7 kDa	Neaiwask	917.4536	917.4606	Mannose-specific lectin precursor from *D. findleyanum*
	Tdgengnyviilqk	1,562.7266	1,562.7940	
	Dgnlvlyskpifatgtnr	1,964.9674	1,965.0320	
24 kDa	Tdgengnyviilqk	1,562.7214	1,562.7940	Mannose-specific lectin precursor from *D. findleyanum*
	Dgnlvlyskpifatgtnr	1,964.9602	1,965.0320	

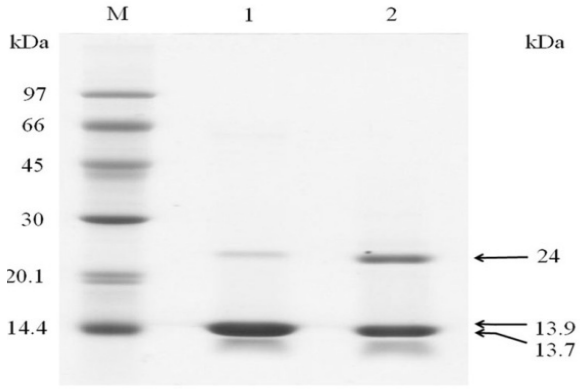

Fig. 2. Reducing gradient SDS-PAGE analysis of mannose-binding proteins purified by using mannan-agarose column chromatography; M= molecular mass standard markers, 1= mannose-binding protein of *D. chrysotoxum* (Northeast), 2= mannose-binding protein of *D. chrysotoxum* (North)

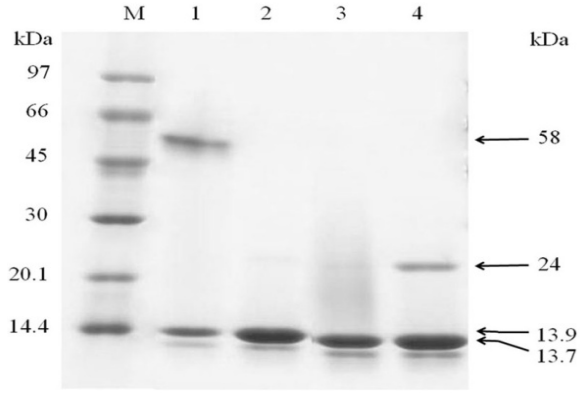

Fig. 3. Reducing gradient SDS-PAGE analysis of the effect of 2-mercaptoethanol and heat on the molecular form of mannose-binding proteins; M= molecular mass standard markers, 1 and 2= mannose-binding protein of *D. chrysotoxum* (Northeast), 3 and 4= mannose-binding protein of *D. chrysotoxum* (North), 1 and 3= mannose-binding protein without heat in the presence of 2-mercaptoethanol, 2 and 4= mannose-binding protein heated in the presence of 2-mercaptoethanol.

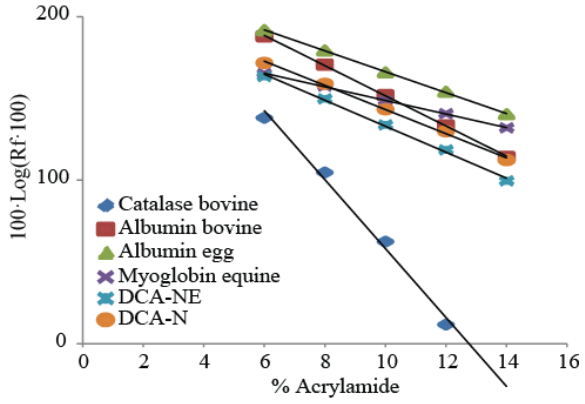

Fig. 4. Ferguson plots for molecular mass standard markers, DCA-NE and DCA-N. Logarithm of relative migrations [100 x Log (*Rf* x 100)] were determined from native-PAGE and plotted against acrylamide concentration.

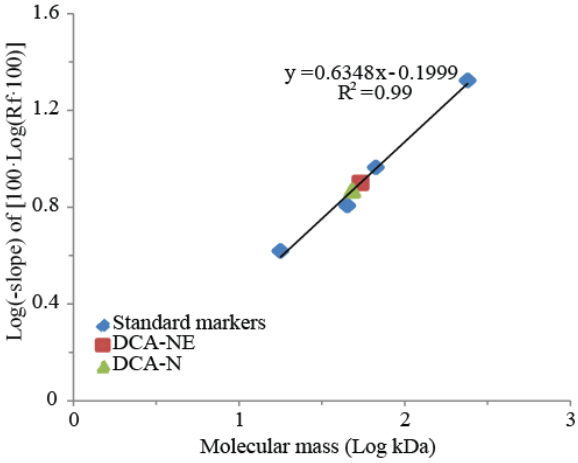

Fig. 5. Standard curve for molecular mass determination of DCA-NE and DCA-N. Logarithm of minus slope was plotted against logarithm of molecular mass of the standard markers. Logarithm of minus slope of DCA-NE, and DCA-N were used to calculate their molecular masses.

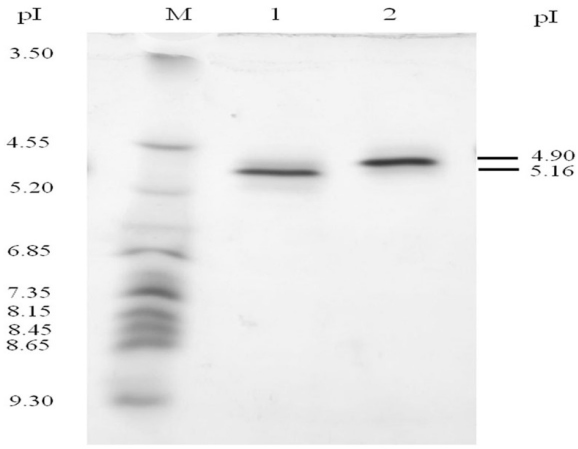

Fig. 6. Determination of pI of *D. chrysotoxum* agglutinin using isoelectric focusing; M= pI markers, 1= DCA-NE, 2= DCA-N.

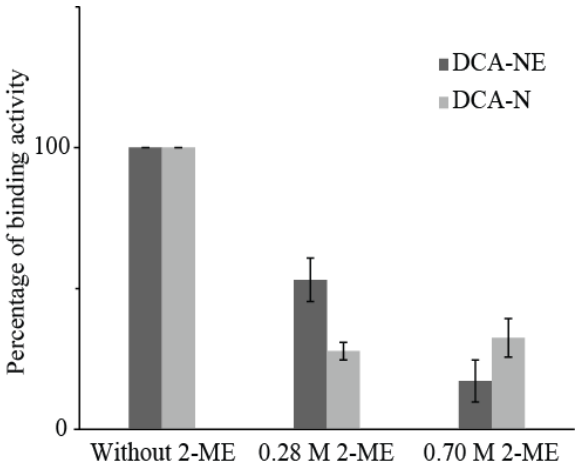

Fig. 7. Determination of effect of 2-mercaptoethanol on binding activities of DCA-NE and DCA-N using solid-phase method; 2-ME= 2-mercaptoethanol.

IEF: The pI of native form of DCA-NE or DCA-N was determined by using IEF method (Fig. 6). Both DCA-NE and DCA-N showed single band at pI of 5.16 and 4.90, respectively.

Effect of 2-mercaptoethanol on binding activity of the lectins: 2-Mercaptoethanol inhibited the binding activities of DCA-NE and DCA-N towards the carbohydrates moiety of HRP (Fig. 7). The activity of DCA-NE decreased slower than that of DCA-N when the concentration of 2-mercaptoethanol increased. In the presence of 2%, and 5% of 2-mercaptoethanol, the remaining activities were approximately 53.11% and 17.20% for DCA-NE, and 27.85% and 32.58% for DCA-N, respectively.

Carbohydrate specificity of the lectins: The carbohydrate specificities of DCA-NE and DCA-N were determined by observing the inhibitory effects of simple sugars on binding activities of the lectins (Fig. 8). Increasing concentration of D-mannose, D-galactose, D-glucose, and D-xylose resulted in a decrease of the binding activities. In contrast, D-ribose showed no inhibitory effect on the activities of both lectins. The most potent inhibitor was D-mannose, followed by D-galactose. The D-mannose, and D-galactose showed 50% inhibition at 7 mM and 78 mM for DCA-NE (Fig. 8A), and at 20 mM and 48 mM for DCA-N (Fig. 8B), respectively. Therefore, both DCA-NE and DCA-N could bind to different structural monosaccharides, but they showed higher specificity towards D-mannose than other sugars.

Deduced amino acid sequences of the lectin precursors: From the DNA sequencing results, 703 bp was obtained for cDNA of each lectin. Each cDNA contained 498 bp ORF which yielded 165 amino acid residues with calculated theoretical molecular mass of 18.58 kDa (Figs. 9 & 10). The proteins containing 165 amino acid residues were named DCA-NE precursor and DCA-N precursor. By using SignalP 3.0, the putative processing site for signal peptide sequence in DCA-NE precursor and DCA-N precursor was between residue 24 and 25 (between A and D) which was in good agreement with *D. officinale* agglutinin precursor (Chen *et al*., 2005b) and *D. findleyanum* agglutinin precursor (Sattayasai *et al*., 2009). Therefore, both lectin precursors should contain the signal peptide sequences of 24 amino acid residues at the N-terminus.

Homology analysis of the lectin precursors: Protein BLAST analysis showed high homology of DCA-NE precursor and DCA-N precursor with lectin precursors from other *Dendrobium* species. By using ClustalW2 program, DCA-NE precursor and DCA-N precursor showed 92% and 90% amino acid sequence similarities with DFA precursor, and 89% and 87% similarities with DOA precursors, respectively. The two lectins of *D. chrysotoxum* revealed 98% similarity at both nucleotide sequence and deduced amino acid sequence in ORF regions. Sugar-binding site analysis according to Barre *et al*., (1996) showed that DCA-NE precursor and DCA-N precursor contained the first and the third mannose-binding sites (QDNY) which were the same as those of many lectins obtained from other *Dendrobium* species. The second mannose-binding site was more variable in which the QDDF residues were changed from QDNY (Fig. 11).

DNA sequencing and comparison of ITS regions: The PCR product of *D. chrysotoxum* (North) approximately 750 bp in length was obtained using the ITS1 and ITS2 primers (Tsai *et al*., 2004) constructed from conserved regions of 18S and 26S rDNA. The PCR product was purified and sequenced with the same primer pairs as those used for PCR. The boundaries of the ITS1, ITS2, and nuclear rDNA coding regions were determined by comparison with several published sequences from other *Dendrobium* species (Tsai *et al*., 2004). The PCR product consisted of the end of 18S rDNA, the ITS1 region, the 5.8S rDNA, the ITS2 region, and the beginning of 26S rDNA. Then, the ITS regions (ITS1, 5.8S, and ITS2) of *D. chrysotoxum* (North) (Fig. 12), *D. chrysotoxum* (Northeast), other *Dendrobium* species and one outgroup species were aligned. The alignment of these sequences showed that 5.8S rDNA was highly conserved, but the ITS1 and ITS2 regions were more variable (data not shown).

Genetic distance and phylogenetic tree obtained from the ITS sequence data: By using the Kimura's two-parameter model, the genetic distances among fifty nine taxa ranged from 0.00 to 0.31 and those among fifty eight *Dendrobium* species ranged from 0.00 to 0.28. The outgroup showed moderate divergence from the *Dendrobium* species with genetic distance ranging 0.22 to 0.31 (data not shown). The phylogenetic tree obtained from the Neighbor-joining method is shown in Fig. 13. According to the tree, the outgroup, *B. blepharistes*, was divergent from the *Dendrobium* species, so it was a suitable outgroup. *D. chrysotoxum* (Northeast) and *D. chrysotoxum* (North) were in the same group with 100% supported by bootstrap test. However, the genetic distance between *D. chrysotoxum* (Northeast) and *D. chrysotoxum* (North) was 0.04 which was a higher value than those among many *Dendrobium* species, such as between *D. flexicaule* and *D. officinale* (0.02), *D. lohohense* and *D. salaccens* (0.02), *D. nobile* and *D. tortile* (0.02), or *D. heroglossum* and *D. linawianum* (0.01).

Discussion

Dendrobium chrysotoxum, native to the Northeastern region and that native to the Northern region of Thailand, show both common and different morphological data; *D. chrysotoxum* from the Northeast has very similar morphology to the species described by Cullen (1992) while the plant from the North has very similar morphology to the species described by Schettler (2006). It is not clear whether the differences are the result of genetic variations, or different adaptations to distinct geographies without genetic modification. Therefore, the molecular data are necessary to address the question. We have been interested in GNA-related lectins since they have many interesting properties (Van Damme *et al*., 1994; Chen *et al*., 2005a; Sudmoon *et al*., 2008; Sattayasai *et al*., 2009; Charungchitrak *et al*., 2011; Upadhyay *et al*., 2011). A GNA-related lectin from *D. findleyanum* (DFA) and its properties were reported by our group. Therefore, biochemical properties of lectins from *D. chrysotoxum* were studied in this work to obtain the molecular data.

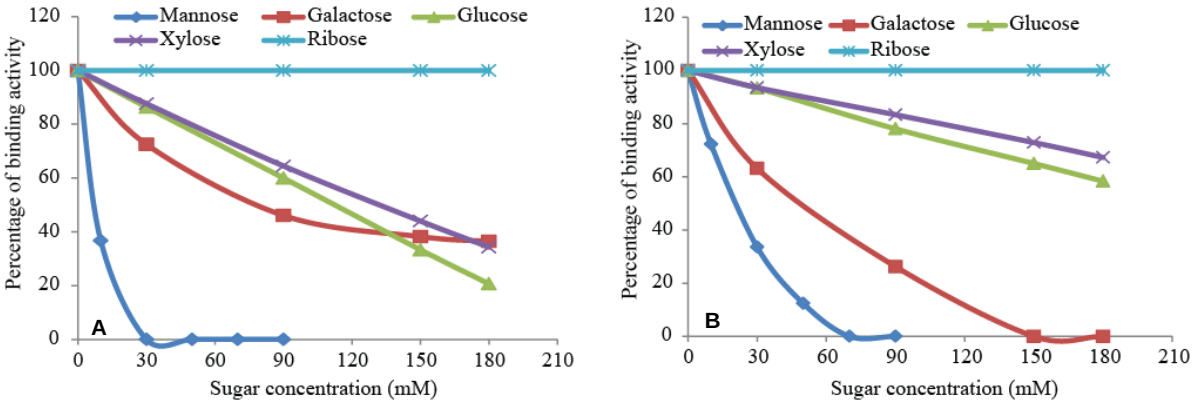

Fig. 8. Binding activities of *D. chrysotoxum* agglutinins in the presence of D-mannose, D-galactose, D-glucose,D- xylose, and D-ribose by using solid-phase method; A= binding activity of DCA-NE, B= binding activity of DCA-N.

```
                                                                M  A  F
1     cacccaagtagagtacaaccaagaacaagctagatagcttcccaacaagccatggctttc  60
      S  I  S  S  T  M  I  F  L  L  S  I  A  L  F  S  T  L  V  S
61    tccatcagctcgacaatgatcttcctcctctattgcgctctttagcacgctggtatct  120
      A↓D  N  H  L  L  P  R  E  R  L  N  P  G  D  F  L  K  Q  D
121   gccgacaatcatttactccccgcgagaggctgaacccgggtgatttcctgaagcaggat  180
      R  Y  M  L  I  M  Q  E  D  C  N  L  V  L  Y  N  L  N  K  P
181   cgatacatgttgatcatgcaagaagactgcaacctcgtcctctacaatctcaacaaacct  240
      V  W  A  S  Q  T  A  N  R  G  S  R  C  F  V  T  L  Q  P  D
241   gtgtgggcatcgcagacagctaatcgaggctcacgttgtttcgtcaccttgcaacccgat  300
      G  D  F  V  I  F  D  E  R  E  G  R  N  E  A  I  W  A  S  K
301   ggcgactttgtcatctttgacgagcgcgaggggcgtaacgaagctatttgggccagtaag  360
      T  D  G  E  N  G  N  Y  V  I  I  L  Q  K  D  G  N  L  V  I
361   accgatggcgaaaatggaaactatgttatcatcctccagaaagatggcaatttggtcata  420
      Y  S  K  P  I  F  A  T  G  T  N  R  F  G  S  T  A  V  V  V
421   tatagtaaaccaatctttgcaaccggcaccaataggttcggctctactgctgttgttgtt  480
      A  K  R  N  R  K  A  H  F  G  V  E  Q  N  I  I  E  V  T  T
481   gccaagcgcaaccgcaaggcgcactttggtgtggagcagaacattattgaagttactact  540
      N  L  *
541   aatctctaaagttgtggcagaggatgttggcctgttgcgtacgatgttaggtagagaataa  600
601   gaccttaatataaccttaaattagtagagtattgcagaggccgcacgcggggatggaatc  660
661   aggcatgccttcatggttggtttgtgtacgtacgagttggttg  703
```

Fig. 9. The cDNA sequence which has been submitted to the GenBank database under accession number KC413408, and its deduced amino acid sequence in ORF region of DCA-NE gene. The start codon (ATG) is bolded, the stop codon (TAA) is bolded and italicized. The putative processing sites for N-terminal signal peptide sequence (between A and D) is indicated by the arrow. Twenty-four amino acid of N-terminal signal peptide is underlined.

```
                                                                M  A  F
1     cacccaagtagagtacaaccaagaacaagcttgatagcttcccagcaagccatggctttc  60
      S  I  S  S  T  I  I  F  L  L  S  I  A  L  F  S  T  L  V  S
61    tccatcagctcgacaataatctttctcctctattgcgctctttagcacgctggtatct  120
      A↓D  N  H  L  L  P  R  E  R  L  N  P  G  D  F  L  K  Q  D
121   gccgacaatcatttactccccgcgagaggctgaacccgggtgatttcctgaagcaggat  180
      Q  Y  M  L  I  M  Q  E  D  C  N  L  V  L  Y  N  L  N  K  P
181   caatacatgttgatcatgcaagaagactgcaacctcgtcctctacaatctcaacaaacct  240
      V  W  A  S  Q  T  A  N  R  G  S  R  C  F  V  T  L  Q  P  D
241   gtgtgggcatcgcagacagctaatcgaggctcacgttgtttcgtcaccttgcaacccgat  300
      C  D  F  V  I  F  D  E  R  E  G  R  N  E  A  I  W  A  S  K
301   tgcgactttgtcatctttgacgagcgcgaggggcgtaacgaagctatttgggccagtaag  360
      T  D  G  E  N  G  N  Y  V  I  I  L  Q  K  D  G  N  L  V  I
361   accgatggcgaaaatggaaactatgttatcatcctccagaaagatggcaatttggtcata  420
      Y  S  K  P  I  F  A  T  G  T  N  R  F  G  S  T  A  V  V  V
421   tacagtaaaccaatctttgcaaccggcaccaataggttcggctctactgctgttgttgtt  480
      A  K  R  N  R  K  A  H  F  G  V  E  Q  N  I  I  E  V  T  T
481   gccaagcgcaaccgcaaggcgcactttggtgtggagcagaacattattgaagttactact  540
      N  L  *
541   aatctctaaagttgtggcagaggatgttggcctgttgcgtacgatgttaggtagagaataa  600
601   gaccttaatataacctcaaattagtagagtattgcagaggccgcacgcggggatggaatc  660
661   aggcatgccttcatggttggtttgtgtacgtacgagttggttg  703
```

Fig. 10. The cDNA sequence which has been submitted to the GenBank database under accession number KC413409, and its deduced amino acid sequence in ORF region of DCA-N gene. The start codon (ATG) is bolded, the stop codon (TAA) is bolded and italicized. The putative processing sites for N-terminal signal peptide sequence (between A and D) is indicated by the arrow. Twenty-four amino acid of N-terminal signal peptide is underlined.

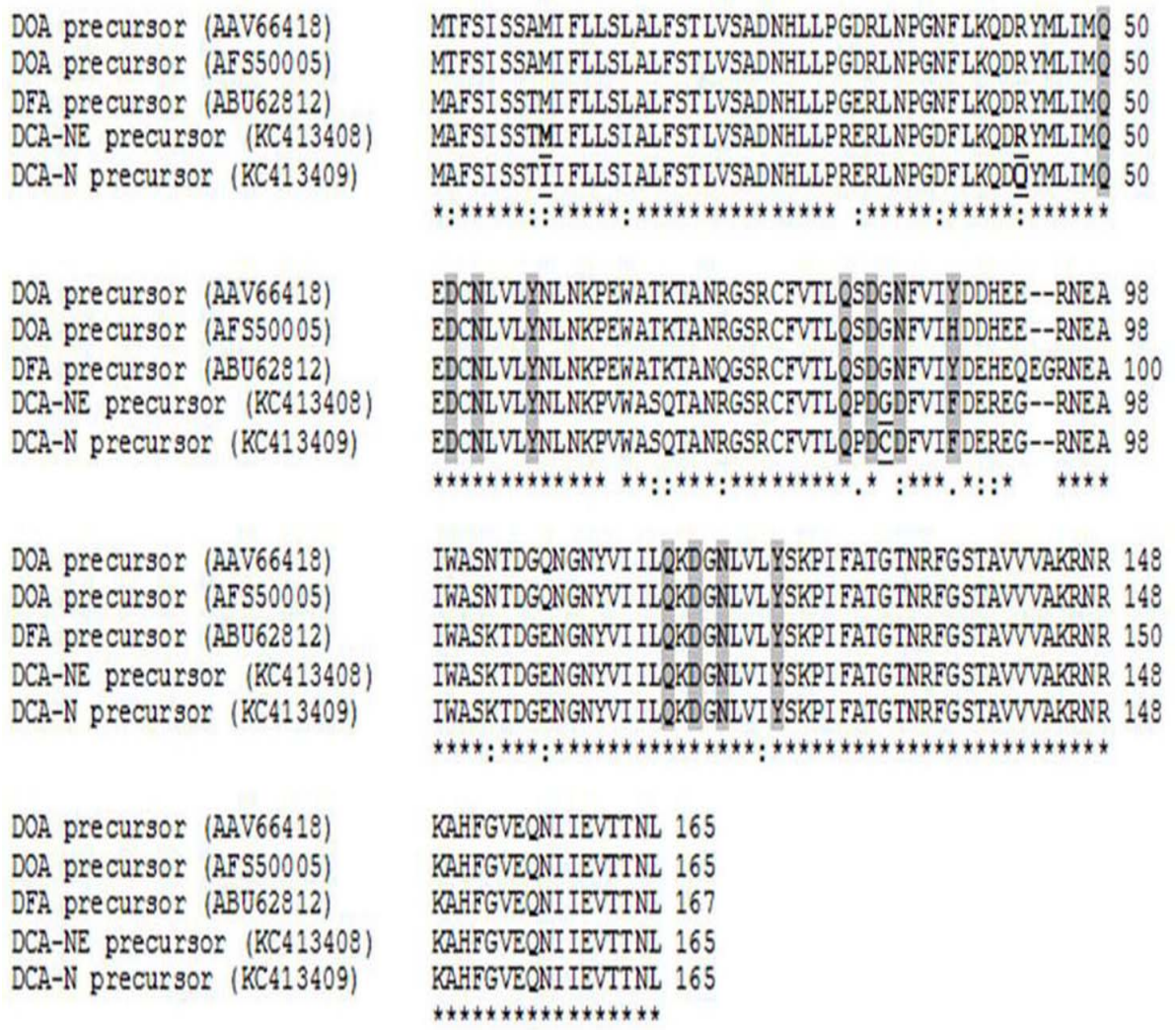

Fig. 11. Alignment of deduced amino acid sequences of DCA-NE precursor and DCA-N precursor with those from *Dendrobium* species (DOA precursors and DFA precursor). The three mannose-binding sites are shaded. The different amino acid residues between DCA-NE precursor and DCA-N precursor are bolded and underlined (at residues 9, 44 and 84). Dashes (-) are introduced for maximal alignment. Residues in any column which are identical in all sequences are shown (*), conserved substitutions are indicated (:), and semi-conserved substitutions are shown (.). GenBank accession numbers are shown in parentheses.

TCGAGACGAAATACACGAGCGATCTTGAGAACCCATCAAAACAAGCAGGGCTAATGCTGC 60
TGCAGAAAAAATTCATCCCAGTCGGTGCCTCATCCCAACCTTGGGGTGGGGACGTGACAA 120
AGGATGGACGAACCCACAAACCGGCGCAGCGTCGCGCCAAGGGATTATCGAAACATGAGA 180
CCTAAAATAGGTTTTGTGGCTTGGGGTGCTGCTGCACGCCATATGGATTGGCACGACTCT 240
CGGCAATGGATATCTCGGCTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACGTGGT 300
GCGAATTGCAGAATCCCGCGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAGGCCAA 360
TCGGCCAAGGGCACGTCTGCCTGGGCGTCAAGC*GTTACGTCGCTCCGTGTCCAGCCACCC* 420
GTCGATGGATGGGCTGGCGATGGCTCGGATGTGCACAGTGGCTCCTCGTGCCCCTCGGCA 480
CGGCGGGCTTAAGAGCGGGTAATCATCTCGTTGGCTGCGAACAATATGGGGTGGATAAAT 540
GCGATGCTCTATGATATTGTGCCGTGCATGCCCGAGAGATGATCATACTTATTCAGGTGA 600
TCCCAAACCATGCGTCGATCCATGGATGGCGTTTTGAAT 639

Fig. 12. ITS region of *D. chrysotoxum* (North) which has been submitted to the GenBank database under accession number KC413407. ITS1 sequence is bolded, 5.8S sequence is underlined, and ITS2 sequence is italicized.

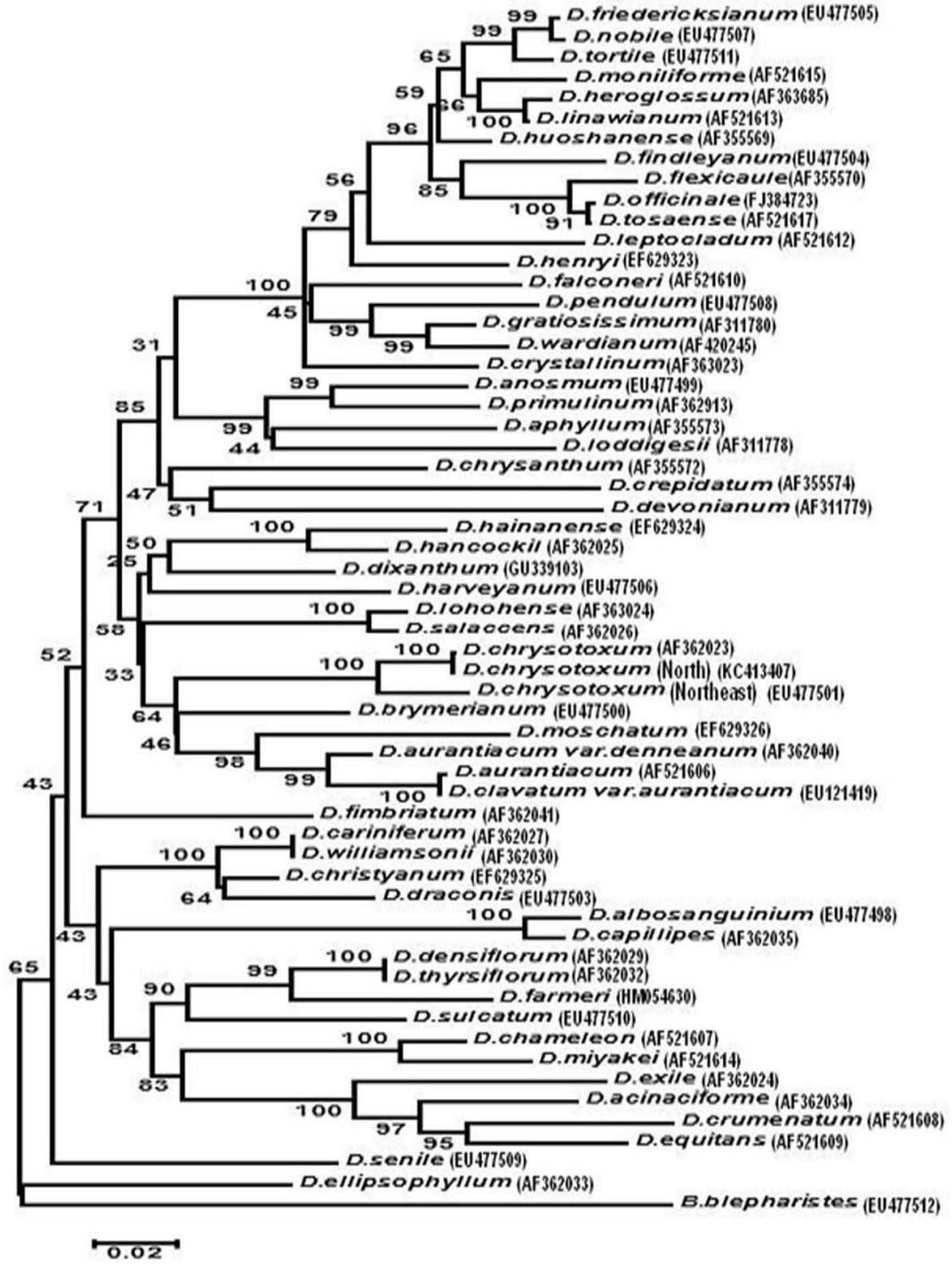

Fig. 13. Neighbor-joining tree, constructed from sequence comparisons of the ITS region, of the 58 *Dendrobium* species and one outgroup species, *B. blepharistes*. Numbers at node indicate level of bootstrap value (expressed as percentage of 1,000 replicates). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances which were used to construct the phylogenetic tree. Scale bar indicates 0.02 substitutions per nucleotide position. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. GenBank accession numbers are shown in parentheses.

The result of SDS-PAGE showed that mannose-binding proteins from pseudobulbs of *D. chrysotoxum* (Northeast) and *D. chrysotoxum* (North) could be purified by using mannan-agarose column. The mannose-binding proteins of *D. chrysotoxum* from the two regions had similar molecular masses of the polypeptide chains on SDS-PAGE, but they had different reactions with 2-mercaptoethanol. In the presence of 2-mercaptoethanol and heat, mannose-binding protein from *D. chrysotoxum* (Northeast) showed one major band at 13.9 kDa while *D. chrysotoxum* (North) had two major bands at 13.7 kDa and 24 kDa. However, the sequence tags of the three bands, obtained using LC-MS/MS, were identified as parts of the DFA precursor (Fig. 2, Table 2). Therefore, the 24 kDa was suggested to be a dimer of the 13.7 kDa band. The aggregation of the proteins in the presence of 2-mercaptoethanol and heat is unusual, but it was reported by many groups (Wolf & Tamura, 1969; Catsimpoolas *et al*., 1970; García-Ortega *et al*., 2005; Broersen *et al*., 2006). In the presence of 2-mercaptoethanol without heat, the major band at 58 kDa from *D. chrysotoxum* (Northeast) were assumed to be the remaining tetramer. These assumptions were proven by using IEF method which showed single band of the purified proteins of *D. chrysotoxum* (Fig. 6). Both lectins showed different levels of hemagglutination activity; mannose-binding protein from *D. chrysotoxum* (Northeast) caused visible hemagglutination at the amount lower than that from *D. chrysotoxum* (North). Therefore, the mannose-binding protein from *D. chrysotoxum* native to the Northeastern region and that from the plant native to the Northern region were named *D. chrysotoxum* agglutinin-Northeast (DCA-NE) and *D. chrysotoxum*-agglutinin North (DCA-N), respectively. The molecular masses of DCA-NE and DCA-N obtained from native-PAGE and Ferguson plots were 54.1 kDa and 48.2 kDa, respectively; so the native forms of DCA-NE and DCA-N are homotetramers. The number of subunits is similar to DFA (Sudmoon *et al*., 2008), but is different from the lectins of other orchids (Van Damme *et al*., 1994). The different reactions of DCA-NE and DCA-N with 2-mercaptoethanol on SDS-PAGE led us to study the effects of 2-mercaptoethanol and simple sugars on binding activities of the lectins. Thiol-protecting reagents, such as 2-mercaptoethanol, were necessary for activity of many galectins (Levi & Teichberg, 1985; Whitney *et al*., 1986; Ola *et al*., 2007), and GNA-related lectins (Sudmoon *et al*., 2008). In contrast, 2-mercaptoethanol decreased the binding activities of DCA-NE and DCA-N, with greater effect on DCA-NE (Fig. 7). From inhibition of binding activity, both DCA-NE and DCA-N exhibited greater specificity towards D-mannose than other sugars (Fig. 8). However, D-mannose could bind to DCA-NE better than DCA-N while D-galactose could bind to DCA-N better than DCA-NE.

The full amino acid sequences of the DCA-NE precursor and DCA-N precursor were deduced from cDNA sequences. Each precursor gave the theoretical molecular mass of 18.58 kDa and contained 24 amino acid residues at the N-terminus. The presence of sequence TGT at C-terminal sequence introducing the possible cleavage between G and T (Chen *et al*., 2005b; Upadhyay *et al*., 2011) is not the processing site for these precursors, since sequence TGT could be detected in the sequence DGNLVLYSKPIFATGTNR by LC-MS/MS (Sattayasai *et al*., 2009). However, DCA-NE precursor and DCA-N precursor should have C-terminal processing sites at another site, because removal of only N-terminal signal peptide yielded the theoretical lectins of approximately 16.05 kDa for DCA-NE, and 16.06 kDa for DCA-N which were much larger than the size of monomers obtained from SDS-PAGE. The deduced amino acid sequences indicated that DCA-NE and DCA-N possessed conserved mannose-binding sites (QDNY) at the first and the third sites, and a variable form (QDDF) at the second site (Fig. 11). The mannose-binding site (QDNY) in each subunit of the lectins enables the lectins to bind to mannose-containing glycoconjugates (Barre *et al*., 1996). Two amino acid substitutions at the residues involved in the second mannose-binding sites may cause the alteration of three-dimensional structure, so DCA-NE and DCA-N become less active to D-mannose, compared to DFA (Sudmoon *et al*., 2008). Therefore, containing two instead of three conserved mannose-binding sites may cause DCA-NE and DCA-N having a reduction of affinity for mannose and gaining the ability for binding to other sugars. So, this is the first experimental report to demonstrate the relation of the mannose-binding sites and the binding activities of GNA-related lectins in orchids. In addition, DCA-NE and DCA-N have different amino acid residues in some positions which may cause the divergence of biochemical properties. Amino acid residues at positions 44 and 84, DCA-NE precursor contains R (pI of 10.76) and G (pI of 5.97) while DCA-N precursor contains Q (pI of 5.41) and C (pI of 5.66), respectively; thus, the side chains of R and G should cause higher pI of the DCA-NE. This interpretation is supported by the IEF result (Fig. 6). In addition, the position 84 is in the area of the second sugar binding site. The different amino acid residues at this position may be the cause of different binding activities between DCA-NE and DCA-N (Fig. 8).

To obtain more molecular data, ITS sequence which was widely used as a tool for identification of species or varieties was used to compute the genetic distances and construct the phylogenetic tree (Fig. 13). The genetic distance between *D. chrysotoxum* from the two regions had a higher value than those among many *Dendrobium* species; although, they were in the same group of the tree. The result indicates much genetic variation between them. In addition, *D. chrysotoxum* native to the North is very close to *D. chrysotoxum* reported by Xu *et al*., (2006); the two orchids should be the same plant.

Conclusion

Based on the differences in biochemical properties and deduced amino acid sequences of the GNA-related lectins, the genetic distances and the phylogenetic tree of ITS sequences of *D. chrysotoxum* native to the Northeastern region, and that native to the Northern region of Thailand, we argue that these two ecotypes should be placed at least two different varieties of the same species if not as to two different species of genus *Dendrobium*. Therefore, the difference in morphological character between the orchids of the two regions must be at least partly result from genetic variation, not from

different adaptations to distinct geographies without genetic change. Since the pseudobulb of *D. chrysotoxum* which has been used in traditional Chinese medicine contains many novel chemical compounds (Ng *et al*., 2012), *D. chrysotoxum* native to the Northeastern region of Thailand may have active substances different from the orchid native to the Northern region of Thailand or China. Therefore, accurate identification of the species is necessary for clinical applications.

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