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Proteins as Biomarkers for Taxonomic Identification of Traditional Chinese Medicines (TCMs) from Subsection *Rectae* Genus *Clematis* from China

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Abstract: Proteins are expressed form of genome which can be biomarkers for identification of botanic drugs. Two dimensional gel electrophoresis of leaf protein was used to know the phylogenetic relationship between species of subsection Clematis and subsection Rectae of the genus Clematis (Ranunculaceae). A protocol was optimized for leaf proteome analysis of Clematis species. Proteins were precipitated by three different extraction procedures and Phenol-SDS method (PSM) produced good results (2.35±0.345 mg/g). Proteins were detected by silver staining and master maps were compared by Melanie 3.0 software package. Protein spots showed pI and Mr in-between 3.0 to 10.0 (pH) and 7 kDa to 70 kDa, respectively. A high level of polymorphism was depicted among the 1085 spots scored: only 255 spots were common to all. The mean number of alleles per locus was only two. The results predicted that nine proteins were solely present in C. chinensis when compared with C. finetiana and C. armandii. Thirteen proteins in C. finetiana (0.75-0.95 fold) and twelve proteins in C. armandii (1.05-1.66 fold) were up-regulated and seven proteins in former (0.66-0.94 fold) and three proteins in latter (1.07-1.20 fold) were down regulated in comparison with C. chinensis. Similarity Index Value (SIV) of selected common/differential was calculated to make proteomic cluster tree (PCT) by hierarchical cluster approach with average linkage using the program MVSP. The genetic distance (GD) 0.4~0.45 and 0.25~0.55 was observed at intra-subsection level in Clematis and Rectae. In the analysis, C. chinensis showed different patterns of protein bands, representing the different genes/allelomorphs. The efficiency and applicability of proteomic approach as taxonomic tool and general properties of the identified proteins are presented and discussed.

Key words: Traditional chinese medicine · Clematis chinensis · Two-dimensional electrophoresis · Leaf proteome · Taxonomy · Botanic drugs · Genus Clematis

INTRODUCTION

Plants play a vital and key role in human life for food subsistence and medicaments. In China, mostly Traditional Chinese Medicines (TCMs) comprise of herbs and their products. These TCMs have been used in many countries for centuries [1, 2]. Even now, there is an increasing trend for the global use of botanical medicines. In general, only a few markers or active components are employed for evaluating the quality and authenticity of TCM [3]. Usually, TCMs have a composition of a diversity of ingredients and their contents vary with not only the cultivar but also with growing conditions based on geographical origins, harvest time, processing methods and storage duration [4]. Hence, it is hard to maintain quality of TCMs by merely relying on traditional taxonomic methods.

The genus *Clematis* (Ranunculaceae) comprises of more than 300 species worldwide, including 147 (93 endemic) in China [5] and it is medicinally very

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important because many of its plant species are used in different TCMs and other herbal pharmacopeias [6-8]. *Clematis chinensis* which belongs to subsection *Rectae* of genus *Clematis* (Ranunculaceae) is very significant plant because it has been used in many TCMs to cure biliary tract disorders [7], anti-tumor [8], anti-inflammatory [1], cardiovascular protective [9], hepatic protective [10], analgesic and diuretic [2]. The infrageneric classification of the genus *Clematis* (Ranunculaceae) has pending definite phylogenetic analysis and it is still uncertain that some species are placed in different sub-genera in classical classification approaches [11]. As traditional classifications primarily rely on floral characters for the major divisions of the genus [12] which cant distinguish each and every species clearly. Some species of subsection *Clematis* and subsection *Rectae*; subsection *Connatae* and subsection *Crispae* are morphologically so closely related to each other that it is difficult to identify them and ascertain their systematic position [13] which causes hindrance in QC of TCMs.

Recently many molecular level approaches such as RAPDs, RLFPs, single tag sequences (STS), 1-DE and 2-DE are being employed in phylogenetic study of different taxa. 2-DE has proven to be a powerful tool for analyzing complex mixtures of proteins [14]. The resolving power of 2-DE as separation technology has found great utility in proteomics studies of plants [15]. 2-DE has been employed for assessing genetic variability and for establishing genetic distances and phylogenetic relationships between lines, species and genus [16, 17]. If two genotypes share identically expressed structural genes, they will depict spots located at the same position on the gels. If the structural genes differ in such a way that the encoded polypeptides have different isoelectric or apparent molecular masses, the two spots will appear at different places [18]. The intensity of spot revealed on 2-D gels depends on the quantity of protein. Thus, if two genotypes differ in the accumulation of common protein, the difference can also be observed on 2-D gels. The automated quantification of spot intensity is necessary for precise estimation of these variations [19] but these quantitative variations can also result in presence/ absence of corresponding spots. Proteomic approach has been extensively used to establish genetic relationships between species [20], among genera [21]. In 1995, distance indices in comparison between different genomes of tribe *Triticeae* were analyzed by using 2-DE approach [18]. In another research, genetic relationships among various genera of family Brassicaceae were explored by using 2-DE methodology. They calculated the genetic distances (GD) on basis of common and distinct spots. They declared that comparative proteomic approach may be helpful in shorten of the transfer between model and agronomic target species [22]. Maria *et al*. in 2005 has differentiated two varieties of green coffee by using 2-DE approach and they were able to determine that sixteen proteins were solely present in *Coffea canephora* and five were found in *Coffea arabica*. And eight proteins were up-regulated in *Coffea arabica* and one protein was down-regulated in *Coffea canephora* [23]. Ginseng specimens were identified by proteomic approach for its quality control (QC) [24] and populations of different geographical origins were isolated on basis differential patterns of proteins [25, 16]. In past studies no attempts were made to use 2-D maps for phylogenetic analysis of genus *Clematis* to classify the different TCMs species *via* polypeptide chains present in only single species and/or up- or down-regulated in various species. We first time have optimized a protocol for leaf proteome of the genus *Clematis* and, differential proteomic patterns were revealed as biomarkers for characterization of different species of genus *Clematis* (@ sub-sectional level). From taxonomic point of view, this study is of paramount significance because it will not only help in taxonomy but also in QC of TCMs of this genus and other plant taxa.

MATERIALS AND METHODS

Plant Material: Seven plant species representing section *Clematis* (genus *Clematis*) were collected from Tian Mu Shan Biosphere Reserve (TMSBR), Zhejiang, China. Their herbaria numbers and geographical distribution details are presented in Table 1. One species *Anemone flaccida* was used as out group. Fresh leaves of same age were collected from each species for proteomic analysis. Only leaves without any stress symptoms were selected, washed with dist. water, blot dried and stored at -80°C until extraction. Herbarium specimen of each species was prepared and placed in the herbarium of Department of Chinese Medicine and Engineering, Zhejiang University, Hangzhou, China.

Chemicals and Materials: Mineral oil, Bisacrylamide(bis), Tris (hydroxymethyl) aminomethane (Tris), Sodium dodecyl sulfate (SDS), Glycine, N,N,NU,NU-tetramethylethyldiamide (TEMED), Ammonium persulfate (APS), Glycerol, ultra pure Urea, protease inhibitor cocktail, 2-D cleanup kit, 2-D Quant Kit were purchased from Amersham Pharmacia Biotech. Acrylamide, Dithiothreitol (DTT), 3-3-1-propane-sulfonate (CHAPS),

Table 1: Plant species included in the analysis; voucher number & geographical distribution

Codes	Species	Herbaria Number	Classification (Wang W.T.2005)	Geographical distribution and habitat information
D.	*C. finetiana* Level. et. Vant	Zh. 712111	(Clematis: Rectae)	Tian Mu Shan Biosphere Reserve
N.	*Clematis chinensis* Osbeck	Zh. 71211	(Clematis: Rectae)	Tian Mu Shan Biosphere Reserve
Q.	*C. armandii* Franch	Zh. 71216	(Clematis: Rectae)	Tian Mu Shan Biosphere Reserve
L.	*C. ganpiniana* (Level. et Vant.) Tamura	Zh. 71217	(Clematis: Clematis)	Tian Mu Shan Biosphere Reserve
I.	*C. apiifolia* DC	ZH. 71214	(Clematis: Clematis)	Tian Mu Shan Biosphere Reserve
P.	*C. argentilucida* (Level. et Vant.) W.T. Wang	Zh. 712113	(Clematis: Clematis)	Tian Mu Shan Biosphere Reserve
A.	*C. peterae* Hand- Mazz	Zh. 712112	(Clematis: Clematis)	Tian Mu Shan Biosphere Reserve
W.	*Anemone flaccida* Fr. Schmidt	Zh. 712114	(Anemonanthea)	Tian Mu Shan Biosphere Reserve

Coomassie G-250 (ultra pure grade) and Agarose were obtained from Shanghai Biotech. Iodoacetamide (IAA) was purchased from Fluka BioChemika. HPLC-grade acetonitrile was purchased from Merck, Germany. HPLC-grade trifluroacetic acid (TFA) was purchased from Tedia, USA. Formic acid (FA) was purchased from Acros Organics. All other solvents were of analytical grade. The protean IEF cell, Densitometer, Powerlook 2100XL scanner (Umax, Hanchu, Taiwan), Melanie software package and non-linear immobilize dry strips pH gradient 3-10 (24cm long) were obtained from Amersham Biosciences. Bromophenol blue, carrier ampholytes and agarose were purchased from Pharmacia Diagnostics (Uppsala, Sweden).

Protein Extraction and Sample Preparation: To obtain high resolution and good results by 2-DE approach, optimization of extraction and solubilization methods is inevitable. To analyze the leaf proteome of *Clematis chinensis*, we have used three different methods *viz* Phenol-SDS Method (PSM), TCA Method (TCAM), Lysis Buffer Method (LBM) and selected the one with better protein spots resolutions and fair back ground. Each extract was made of two or three individuals and each accession was represented by three gels form different extracts.

Ph-SDS Method (PSM):

Preparation of Dry Tissue Powder: For protein extraction leaves ca. 5.00g were cut into small pieces by clean scissors and ground in liquid N_2 in a pre-chilled mortar and pestle. The powdered tissue ca. 0.2-0.3 g was re-suspended in 1.0-2.0 mL cold acetone in 1.5 or 2.0 mL microtubes. Then it was vortexed thoroughly for 1min and centrifuged at 10,000 rpm using Eppendorf (Centrifuge 5810 R) for 5 min (4°C). The process was repeated twice. After the initial two washes, the pellet was transferred into a mortar and allowed to dry at room temperature (ca. 20 min). The dried powder was further ground to a finer powder by the aid of quartz sand and then transferred into new microtubes. The powder was sequentially rinsed with cold 10% TCA in acetone until the supernatant became colourless, then it was washed with cold aqueous 10% TCA twice and finally with cold 80% acetone twice. Each time the pellet was re-suspended completely by vortexing and centrifuged. The final pellet was dried at room temperature and used for protein extraction, or stored at -80 °C for future use.

Protein Extraction: Phenol extraction of proteins is based on the protocol described with few modifications [26]. About 0.05-0.1 g of the dry powder of leaf tissue was re-suspended in 0.7 mL phenol (Tris-buffered, pH 8.0; Sigma St. Louis, MO, USA) and 0.7 mL dense SDS buffer (30%sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% 2-mercaptoethanol) in a 2.0 mL microtube. The mixture was vortexed thoroughly for 3 min and phenol phase was partitioned by centrifugation at 10,000 rpm for 10 min. The upper phase (phenol) was pipetted to fresh microtubes (0.2 mL for 1.5 mL tube, 0.4 mL for 2.0 mL tube). Extraction process was repeated and phenol fractions were mixed. To precipitate proteins, about 5 volumes of cold methanol plus 0.1 M ammonium acetate was added to phenol phase and mixture was stored at -20°C for one hour. Precipitated proteins were recovered at 10,000 rpm for 10 min (4°C) and washed with cold methanolic ammonium acetate and cold 80% acetone twice (each). The final pellet was dried and stored at -20°C until use. Prior to 2-DE run, proteins were dissolved in 100uL of lysis buffer containing 7 M urea, 2 M thiourea (w/v), 2% CHAPS, 1% Ampholytes pH 3-10 (v/v), (Biorad), 40mM Tris, 10mM Acrylamide.

TCA-Acetone Method (TCAM): Leaf powder was prepared as mentioned above. Dry powder ca. 5.00g was homogenized in four volumes of cold acetone (-20°C). Extraction buffer containing 10% TCA (w/v) and 0.07% β-mercaptoethanol (v/v) in acetone was used in first

extraction and, 10% TCA (w/v) in acetone was used for second extraction [27]. The sample was kept at -20°C over night and centrifuged at 14,000 rpm for 15 min and pellet was recovered. The obtained pellet was rinsed with cold (-20°C) acetone containing 0.07% (w/v) DTT and centrifuged again. The washing was performed twice. The pellet was vaccum dried and solubilized in lysis buffer (7 M urea, 2 M thiourea, 3% CHAPS, 1% ampholytes (pH 3-10), 40mM Tris, 10mM acryl amide) by carefully sonicating/ vortexing. The obtained slurry was centrifuged again and supernatant was stored at -20°C until use.

Lysis Buffer Method (LBM): Dry powder of leaves was obtained by the same foresaid method. Leaf powder was directly solubilized in the lysis buffer (0.75 mL/ 200 mg) containing 7 M urea, 2 M thiourea, 3% CHAPS, 1% ampholytes (pH3-10), 40mM Tris, 10mM acryl amide [28]. Extraction was allowed for two hours at room temperature under continuous shaking while alkylation was blocked by adding 10mM DDT. Extracts were centrifuged at 10,000 rpm for 20 min using Eppendorf (Centrifuge 5810 R) at 4°C and supernatants were stored at -20°C until further use.

In each case, obtained proteins were purified by 2-D clean-up Kit (Amersham Biosciences) and quantified by the Bio-Rad protein assay (Hercules, CA, USA) with bovine serum albumin (BSA) as standard [29]. Prior to first run, protein extracts were supplemented with trace of bromophenol blue (BPB) and resolved by two-dimensional electrophoresis.

Two-dimensional Gel Electrophoresis (2-DE):

IEF in IPG Strips: The first dimension was performed on IPG-strips (24cm length, 0.5mm thickness) with non linear gradient from pH 3~10 (Amersham Biosciences) [30]. The rehydration solution contained 7 M urea, 2 M thiourea, 3% CHAPS, 1% ampholytes (pH3-10), 40mM Tris, 10mM acrylamide. Purified protein samples were dissolved in rehydration solution supplemented with 0.02% Bromophenol blue and DTT (2.8mg/ml) was added just prior to use. For analytical run (to visualize common and differential proteins) 60µg proteins of each sample were loaded onto dry IEF strips, using the overnight in-gel reswelling method [31]. The re-swelled IPG strips were subjected to IEF at 20°C with first rehydration step for 12 hours at 30 V, followed by focusing for 1 hour at 100 V, 1 hour at 200 V, 1 hour at 500 V, 1 hour at 1000 V, 30 min for voltage increasing to 8000 V and remaining 8000V for 66 kVh on an IPGPhor (Amersham Biosciences).

SDS Phase: Focused strips were equilibrated using a first incubation step in equilibration solution (6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris-HCl, pH 8.8), containing 1% w/v DTT for 15 min, followed by a second incubation step in 2.5% w/v iodoacetamide in the same equilibration solution for 15 min as suggested by Roh [32]. Equilibrated strips were gently rinsed with SDS electrophoresis buffer and loaded on top of 12.5% w/v vertical SDS-polyacrylamide gels (26×20 cm), prepared using a Bio-Rad Mini Protean II system (Bio-Rad, Hercules, CA, USA), according to method of Laemmli [33]. The second dimension separation was performed sequentially with a constant voltage of 5W/gel for 1h, followed by 20W/gel for 6h using the Ettan DALT II system (Amersham Biosciences). A molecular weight calibration kit for SDS electrophoresis (Amersham Biosciences) was used as a molecular size marker on all gels. During the whole temperature was kept constant at 10°C.

Staining of Gel Images: All gels used for analytical purpose were fixed with solution containing 40% ethanol and 10% acetic acid for over night and stained with silver stain for spot visualization and matching [34, 35]. The gels were washed with milliQ water (Millipore Bradford, MA, USA).

Proteins Gel Images and Statistical Analysis: Sliver-stained gels were scanned using Powerlook 2100XL scanner (Umax, Hanchu, Taiwan) and gel images were analyzed using Melanie 3.0 software [36]. In order to minimize the contribution of experimental variations, three separate gels were analyzed for each accession and reference gel was created for each accession. Those spots displaying the same distribution patterns in three replicas were retained in reference gels and selected for further analysis. A standard gel was constructed with highest number of spots (sp. N). All subsequent spot matching and analysis was performed by comparing all gel maps with the standard gel image by the software and manually. Gel patterns from each independent analysis were matched together and the relative abundances (%V) of each spot in gel were compared, using student's *t*-test ($p<0.05$). The percent value (%V) represents the pixel density of each spot normalized for the total pixel density from all spots in the same gel. It has been shown that for a large proportion of proteins, the integrated optical density is linearly related to the protein amount [19, 37]. This internal calibration can make the data independent of gel variations, as recommended by software manufactures

[16]. Marking by a box (□) around the common spots in all gel replicas was also performed to locate quantitative and qualitative variation among different spots of gel images. All those proteins which were up-regulated or down-regulated in the three samples were studied by statistical analysis (student's *t*-test) with the software. The M_rs of proteins was determined by co-electrophoresis of standard marker proteins on the gels (Sigma, St. Louis, Mo, USA). The P*I* of the proteins was calculated by migration of protein spots on 24cm IPG (pH 3-10, non-linear; Amersham Biosciences) strips.

Distance Indices: Considering the presence or absence of each spot in each accession, the data were formulated in form of matrix with as many rows as many spots and as many as columns as number of accessions (830 × 8). The comparisons were performed by counting the number of spots commonly present (n_{xy}) or absent (n_{oo}) and specifically present in one (n_{xo}) or other (n_{oy}) of the two accessions. This binary form of data was used to generate proteome cluster tree (PCT) using Multivariate Software Package (MVSP).

RESULTS

Extraction of Proteins: Leaf proteins of collected sample plants (Table 1.) were extracted by three different protocols; phenol SDS method (PSM), TCA acetone method (TCAM) and lysis buffer method (LBM). The extraction results indicated that starting from ca. 1.0 g of fresh leaf of *Clematis* produced 0.2-3.0 g of dry powder and finally 2.35±0.345 mg proteins were obtained by PSM. The other two methods TCAM and LBM produced 1.8±0.215 mg and 1.2±0.325 mg of proteins, respectively from same amount of Fresh Material (FM) weight (Figure 1).

Two Dimensional Electrophoresis: For comparison of efficiency of extraction methods, for each accession equal amount of precipitated proteins (60µg) were loaded and analyzed by two-dimensional electrophoresis technique. The 2-D maps were stained by silver stain for this comparative study. The obtained 2-DE gel images revealed that 650 spots by PSM, 510 spots by TCAM and 460 spots by LBM were generated. The PSM produced gel image having maximum number of spots with sharp boundaries and fair background. The other protocols gave gel maps with low spot score and high back ground with vertical streaking (Figure 2). There was good repeatability shown by analyzing different species by the optimized protocol (Figure 3).

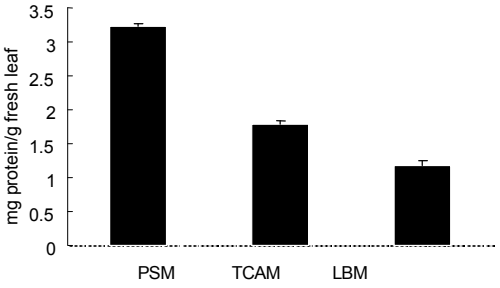

Fig. 1: Protein yield of *Clematis chinensis* leaf by three different extraction methods. Proteins are quantified by the Bio-Rad protein assay. Results (mg protein/g fresh leaf) are the mean ± S.D values ($P < 0.05$) from three independent experiments

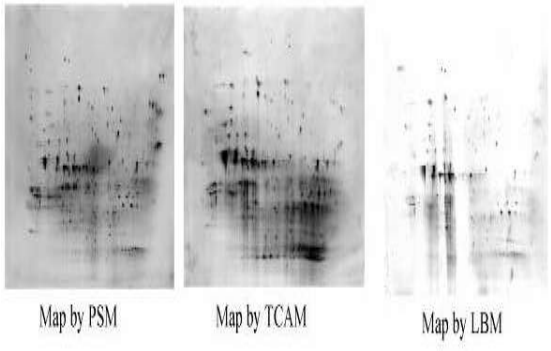

Fig. 2: Three 2-D maps of obtained by three different protocols; PSM: phenol-SDS method, TCAM: TCA acetone method, LBM: Lysis buffer method. The gels were stained with Silver stain

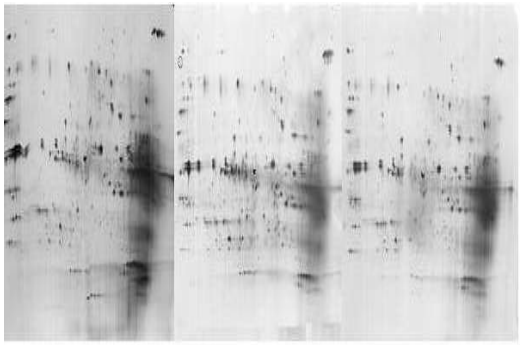

Fig. 3: Three 2-D maps predicting experimental repeatability

Statistical Analysis and Pattern Recognition: All data were expressed as mean ± S.D. Student t-test was used to compare the data of leaf proteome profile of 7 Clematis and one Anemone (out-group) species. The condition of $P<0.05$ was considered to be statistically significant. The simultaneous comparison of a large number of complex objects was facilitated by

Table 2: Similarity index Value (SIV) values of different species of *Clematis* genus:

	%Vol D	%Vol N	%Vol Q	%Vol L	%Vol I	% Vol P	%Vol A	%Vol W
D	1.00	0.67	0.60	0.62	0.58	0.63	0.60	0.28
N	--	1.00	0.54	0.52	0.39	0.57	0.58	0.12
Q	--	--	1.00	0.42	0.36	0.51	0.51	0.24
L	--	--	--	1.00	0.75	0.67	0.58	0.21
I	--	--	--	--	1.00	0.61	0.57	0.45
P	--	--	--	--	--	1.00	0.48	0.33
A	--	--	--	--	--	--	1.00	0.24
W	--	--	--	--	--	--	--	1.00

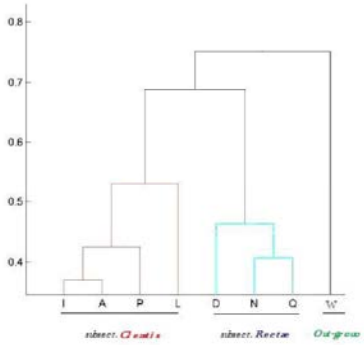

Fig. 4: Dendrogram based on proteomic data depicting phylo-genetic relationship of section *Clematis* (genus *Clematis*), the accessions names are same as given in Table 1.

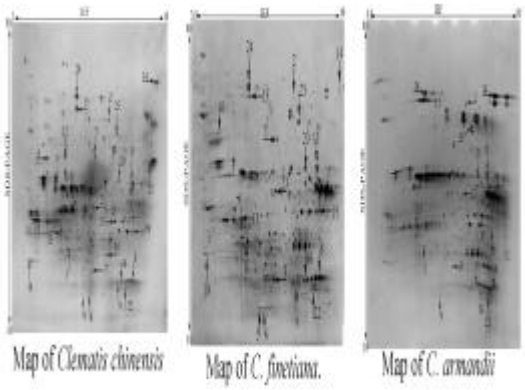

Fig. 5: Representative maps of *Clematis chinensis, C. finetiana* and *C. armandii.* Proteins were resolved using a non linear gradient pH 3-10 in first dimension and 12% SDS-PAGE in the second dimension. Marked spots show differential changes in three species

reducing the dimensionality of the data set via two-dimensional mapping procedures. The resulting data were displayed as "score plots" in form of matrix. The SIV depicted that species D, N, Q are have more affinity with each other than with others. There was a least relationship seen between species N and W (0.12%) as shown in Table 2. Then using software cluster methodology, phylogenetic tree was constructed named as proteomic cluster tree (PCT) by hierarchical cluster approach with average linkage using the program MVSP. Three distinct clusters were formulated clearly differentiating phylogenetic relationship at intra-subsectional. The GD value at intra-subsection level ranged from 0.40~0.45 and 0.30~0.55 in Rectae and Clematis, respectively. There was more GD (0.60~0.68) observed between the two subsections and out group depicted the most GD (0.77) delimiting it as separate branch (Figure 4). It was interesting that the species N and D of showed more genetic affinity with each other as compared with species Q of the same subsect Rectae.

All gel maps showed a broad distribution of protein spots in a pI range from 3.0 to 10.0 and a mass range from 7 kDa to 70 kDa (Figure 5). The three gel pictures depicted differential distribution patterns of spots in three analyzed samples. By making quantitative (IRM) comparison among the accessions it was seen that thirteen proteins (1.05-1.66 fold) in *C. finetiana* and twelve proteins (0.75-0.95 fold) in *C. armandii* were up-regulated while seven proteins (0.66-0.94 fold) in former and three proteins (1.07-1.20 fold) in latter were down-regulated (Figure 5). The qualitative analysis depicted that four proteins were absent in *C. finetiana* and eight proteins were missing in. *armandii*, respectively if compared them with (sp. N) IRM (Figure 5). The Figure 6 summarizes the data of twenty five differential distribution patterns of proteins in three analyzed species of the subsection *Rectae* (genus *Clematis*). It shows relative up- or down-regulation of marked proteins. This indicates that there is a quantitative variation occurring at different allele level in three species of subsect, *Rectae* (Figure 6).

A number of these marked proteins were identified by MALDI-TOF-MS (data is presented in another paper). There are some proteins which demonstrate quantitative and qualitative variation among the various species of Rectae. Eight proteins were solely present in species

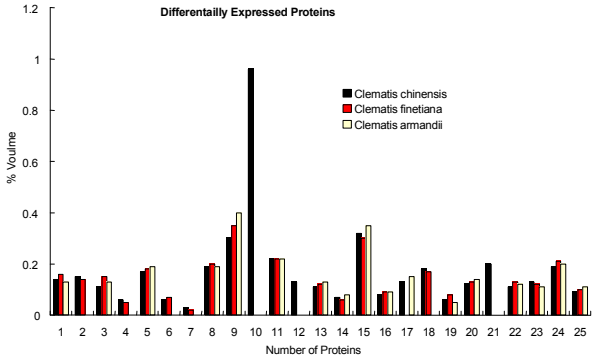

Fig. 6: Summary of differentially expressed proteins in three species, with *Clematis chinensis* as IRM containing all marked proteins.

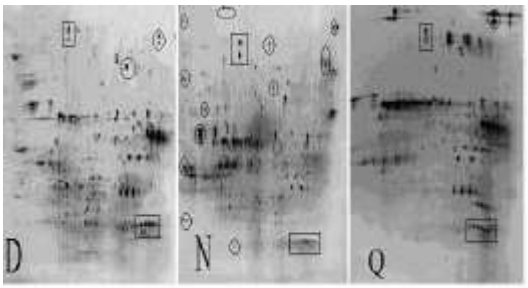

Fig. 7: Spots marked in Box (□) show similar points and spots marked by circle (○) are differential spots presents in three 2-D maps of species (subsect, *Rectae*).

C. chinensis (N) which can be used as biomarkers for its identification from species Q and other four proteins were present in *C. chinensis* only which can be used to identify it from its closest species D (Figure 7).

DISCUSSION

Traditional Chinese Medicines (TCMs) are playing very important role in health and economy of China. There are various attempts made to modernize these TCMs to prove their safety to compete in the western market. Many species of genus *Clematis* are being used in TCMs [38] and different analytical and molecular level attempts are being incorporated to the science day by day [39]. It is inevitable to use precise and accurate methods to ensure the QC of TCMs and 2-DE approach is employed to identify the exact plant species of genus *Clematis*. In 2-DE protocol optimization is key factor to obtain good gel maps. Particularly, protein extraction from plant tissue is often complicatedly due to non-protein contaminants indigenous to the plant, such as organic acids, lipids, polyphones, pigments, terpenes, etc [40]. In conventional methods these contaminants are co-extracted with proteins, which interfere the subsequent analysis results [41] and usually these contaminants are more abundant in green tissues than in young seedlings and etiolated material [40]. In our analysis, out of three extraction procedures used, we have concluded that PSM was better than the other two methods, it might be due to its better power to eliminate contaminants from the sample and secondly proteins are well dissolved in phenol phase and separated as upper layer leaving below contaminants in aqueous phase and, phenol also prevents protein degradation pf proteins due to endogenous proteolytic process [42] basic polypeptides appeared as round shaped spots up to pH 10.0 and it produced more number of spots (650 spots) with fair gel images (Figure 2). Hence, PSM was used in this phylogenetic and biosystematic study of genus *Clematis*.

The standard procedure of sample preparation and 2-DE separation ensured the reproducibility of our proteomic analysis. Based on Melanie 3.0 analysis about 760, 750 and 770 protein spots were detected from three species of *Clematis* with silver staining respectively. Most of the protein spots were distributed in the region of p*I* 4-9 and had molecular weights between 15-70 kDa (Figure 5). The variability of spots observed between accessions (three spp of subsect, *Rectae*, four spp subsect, *Clematis* and one sp of *Anemone*) was very high. Of the 1085 spots scored in this analysis, only 255 were common to all. The similarity index and PCT constructed from these differential expressions of proteins depict that all accessions with same genome merge with each other before being merged with other accessions (Figure 4). In the present study many spots showed within genome variability. Many of these spots were also genome specific, but they did not contribute to the discrimination between the genomes, because they increased distances between members of same genome and as well as between members of other genomes. However, they can be valuable in for phylogenetic studies at lower level. By 2-DE taxonomic delimitations can be assigned not only between species of *Clematis chinensi*s from other species of subsect, *Rectae* (*C. finetiana* and *C. armandii*) but also demarcate these species from taxa of subsect, *Clematis* and congruently differentiate for identification purpose by expressed genome patterns. There are twelve (8+4) differentially expressed proteins (Figure 7 & 8) which can be used to characterize the species *Clematis chinensi*s from its closest taxa. Proteomics approach has been employed in revealing phylogenetic relationship among different species of various genera in past by many taxonomists [17].

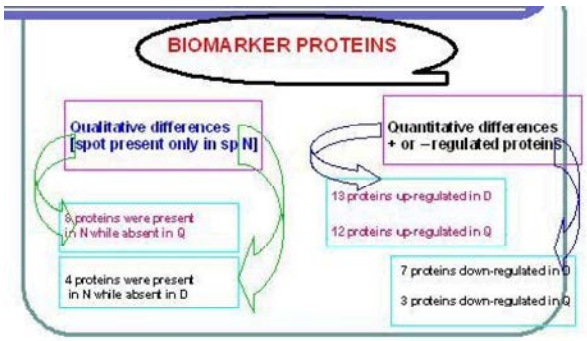

Fig. 8: Pictorial view of quantitative and qualitative variation in protein profile of *C. chinensis* (N) and other species (D & Q) of subsect, *Rectae* (genus *Clematis*).

Within subsect, *Rectae*, the species *C. chinensis*, *C. armandii* and *C. finetiana* genome are related to each other and they form one distinct cluster and species *C. chinensis* is congruently differentiated from other allied species by this technique (Figure 4). Moreover, species of subsect, Clematis are grouped in one cluster with a distinguished genetic distance from each other. The species *Anemone flaccida* (an out-group) is quite well separated in the PCT as a separate line. This analysis proves that 2-DE is reliable and efficient method for demarcation of taxonomic boundaries among various closely taxa which cannot be separated by classical techniques [13]. The relationship found in the present study is in complete agreement with those found by Jonathan and Wang [11, 43]. This study albeit preliminary in nature proves that 2-DE technique can be valuable tool for revealing exact identification and classification of plants that has pertinent significance in quality control (QC) of TCMs and other medicinal pharmacopeias. Exact and accurate identification of herbs is key step towards QC of medicines and food. So, proteomics is reliable and authentic method of identifying the right plant for right use in pharmaceutical and food industry.

CONCLUSION

This is of evidence that proteomic approach can prove to be a good tool for phylogenetic analysis at subsect, level in the genus *Clematis*. Moreover, proteins can be used as biomarkers for identification and classification of different taxa in genus *Clematis* and other genera too.

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