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DETERMINATION OF TAXONOMIC STATUS OF CHINESE SPECIES OF THE GENUS *CLEMATIS* BY USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY (HPLC-MS) TECHNIQUE

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Abstract

A comparative taxonomic study using chemometric and numerical taxonomic approaches on 15 populations of 12 species of major taxa of the genus *Clematis*, belonging to sections, *Rectae, Clematis, Meclatis, Tubulosae* and *Viorna* were analyzed by HPLC coupled with diode array detector and ESI-MS. The chemodiversity profile of saponins proved to be useful taxonomic markers for the genus and results are presented in phenograms. The compound 'Huzhangoside D' was the most abundant in analyzed species of the genus. Numerical taxonomic study was also conducted based on phylogenetically informative characters which corroborated with chemical fingerprinting findings. The significance of chemical markers in taxonomic study as well as their correlation between morphology and chemical compound profile is also debated with its significant role in botanic drugs identification.

Introduction

Clematis L., is the second largest genus of Ranunculaceae, which comprises of more than 300 species worldwide and among these 147 (93 endemic) species are found in China (Wang, 1999). Traditional classification systems are usually based on floral and vegetative characters, and have been used for taxonomic divisions of many plants. Tamura (1966-1968) divided genus *Clematis* into different groups by using morphological characters and similar taxonomic studies have been conducted on various taxa of the genus *Clematis* (Tobe, 1974 & 1980; Tarasevich & Serov, 1986; Snoeijer, 1992; Yano, 1992). However, classification based on characters of seedling and juvenile morphology of *Clematis* species has proved a supporting fundamental in infrageneric classification of the genus (Tamura, 1987) but it is applied to limited level. Some intra and inter genus ambiguities hitherto are present such as some taxa of *Clematis* and *Anemone* are placed in the same tribe (Tamura, 1967; Hoot, 1995).

In China, several attempts based on morphological characters have been carried out to study the phylogenetic position of Chinese *Clematis* (Hua & Li, 2003; 1998; 2000b; 2001; 2002; 2003; 2004a; 2004b; 2004c; Wang & Li, 2005a; Li, 2005b; Yang & Huang, 1992). However, some taxa of the genus; subsections *Clematis* and *Rectae*, and subsections *Connatae* and *Crispae* are so closely related to each other that it is difficult to identify and ascertain systematic position of some species (Wang, 1998).

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Chemotaxonomic studies of sect. *Viorna*, subsect. *Viornae* has been performed using flavonoids distribution patterns (Dannis, 1976). In taxonomic study saponins have been used as chemotaxonomic markers in differentiating various taxa of plants (Michael, 1993). Although, saponins have been isolated from different species of *Clematis* in previous studies (Dekanosidze, 1979; Ulubelen Ayhan, 1970; He *et al*., 2001; Du Zhi *et al*., 2003; Bahcguna, 1989; Baoping *et al*., 1995; Baoping *et al*., 1996; Haruhisa *et al*., 1995; Thapliyal & Bahuguna, 1993; Sudhir & Sati, 1992; Sati *et al*., 1992; Yukio, 2001; Hui *et al*., 2000) but hitherto no attempt has been conducted for chemotaxonomic study of the genus *Clematis* on the basis of saponin profile.

The first aim of the present study was to test different chemical markers to verify whether they may cast light on patterns of systematic relationships within Chinese species of genus *Clematis*. Secondly, role of chemodiversity and chemosystematic study in quality control of herbal medicines is discussed as many Traditional Chinese Medicines (TCMs) are obtained from species of the genus (Ishtiaq *et al*., 2006; 2007; Sun *et al*., 2007).

Material and Methods

Plant specimens: The plant specimens were collected from Tian Mu Shan Biosphere reserve (TMSBR) and Hangzhou (HZ), Wenzhou, Hebei, and Guangdong. The species were identified and their herbaria with voucher numbers were prepared (Table 1). The voucher specimens were deposited in College of Pharmaceutical Sciences (CPS), Department of Chinese Medicine Science, Zhejiang University Hangzhou, China. About 7~10 herbarium specimens of each species were also studied for identification, comparison of morphological features and numerical data generation.

Chemicals and instruments: Acetonitrile and methanol, $CHCl_3$, and n-butanol were of analytical grade (Merck, USA). HPLC grade water was prepared using a Milli-pore water purification system (Millipore, MA, USA). Hand lens, lead pencil, ruler, scanning electron microscope (SEM), light microscope (LM).

Numerical analysis based on phenetic characteristics: For numerical data different specimens freshly collected as well as from herbarium were surveyed by using ruler, hand lenses, SEM, LM. Each and every species data was generated by studying 7~10 fresh or herbarium specimens in order to avoid biased data due to variations in phenetic features that is usually due to variations in environment and in this regard average values of every character were used for matrix formation (Table 2) and to generate phenogram as depicted in Fig 1.

Phytochemical analysis

Extraction: Plant material was air dried in dark at room temperature and powdered. For extraction ca. 1g of powder was refluxed in 20 mL $CHCl_3$ for 1h and filtered. The filtrate was discarded and the residue (plant material) was refluxed again in 40 mL of 50% MeOH for 1hr. Extract was filtered and concentrated at 60°C under vacuum by Buchi rotavapor B-490. The concentrate was dissolved in 5 mL of dist. water and mixed with 7 mL of n-butanol in separatory funnel. After an interval, mixture was partitioned into two layers and under layer was separated and stored as fraction I. The process was repeated once for residue left in funnel and isolated fraction was mixed with first one. The obtained fractions were concentrated and dissolved in 5 mL of MeOH and stored as stock solution at 4°C until use. Each species was represented by three or more specimens and those were extracted and analysed by the same protocol.

Table 1. Plant sources and their geographical distribution and herbarium numbers

Codes species	Herbaria number and wild or cultivated	Classification (Wang W.T.2005)	Geographical distribution habitat information
A- *Clematis peterae* (var) *trichocarpa* W. T. Wang	W, Zh.712112	(*Clematis: Clematis*)	Tian Mu Shan Biosphere Reserve (East TMS)
D- *C. finetiana* Level. et. Vant.	W, Zh.712111	(*Clematis: Rectae*)	Tian Mu Shan Biosphere Reserve (Dong Shan, Shigu)
G- C. *heraclefolia* DC.	W, Zh.71213	(*Clematis: Tubulosae*)	Tian Mu Shan Biosphere Reserve
N- *C. chinensis* Osbeck	W, Zh.71211	(*Clematis: Rectae*)	Tian Mu Shan Biosphere Reserve
Q- C. *armandii* Franch	W, Zh.71216	(*Clematis: Rectae*)	Tian Mu Shan Biosphere Reserve (East Tian Mu Shan)
L- *C. ganpiniana* (Level. et Vant.) Tamura	W, Zh.71217	(*Clematis: Clematis*)	Tian Mu Shan Biosphere Reserve
I- *C. apiifolia* DC.	W, Zh 71214	(*Clematis: Clematis*)	Tian Mu Shan Biosphere Reserve
R- *C. henryi* Oliv	W, Zh.71219	(*Viorna: Connatae*)	Tian Mu Shan Biosphere Reserve
C- *C. intricata* Bunge	W, Zh 712126	(*Clematis: Clematis*)	Hebei Province
T- *C. terniflora* DC	W, Zh 712127	(*Clematis: Rectae*)	Tian Mu Shan Biosphere Reserve
U- *C. huchouensis* Tamura	W, Zh.71212	(*Clematis*: *Viticella*)	Hangzhou, Zhejiang
P- *C. argentilucida* (Level. et Vant.) W.T. Wang	W, Zh.712113	(*Clematis: Clematis*)	Tian Mu Shan Biosphere Reserve

Abbreviations used above: W: Wild; C: Cultivated; Zh: Zhejiang University Herbarium, species are arranged according to classification system of Wang W. T. 2005.

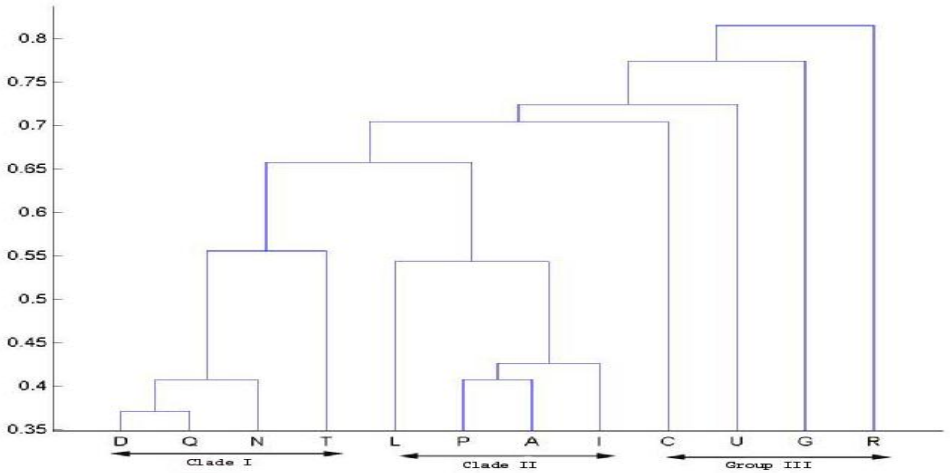

Fig. 1. Phenogram showing affinity relationships among taxa of *Clematis* genus based on morphological characters, as determined by Euclidian distance, average link (Classical "Unweighted Pair-Group Method Using Arithmetic Averages") algorithm. The alphabetical names are same as presented in Table 2.

Sample preparation: The standard compounds ca. 1.5 mg were dissolved in 500 µl of methanol and stored as stock solution at 4°C until use. The stock solution of sample ca. 1.2 mL was centrifuged and used for analytical run in HPLC for each specimen. About 200 µl standard solution was run to calibrate the analytical conditions for experiment.

High performance liquid chromatography coupled with diode array (HPLC-DAD): The HPLC-DAD analysis was carried out on an Agilent 1100 Series HPLC with diode array detector using a 5µm Agilent RP column C_{18} (4.00 mm×250 mm). The column temperature was maintained at 30°C. Optimum detection wavelength was 204-208 nm. A binary gradient elution: acetonitrile (A) and 0.1% aqueous formic acid (B) was used. The mobile phase flux conditions; 0-5 min, 5-10% A; 6-18 min, 10-17% A; hold isocratic elution for 10 min; 29-60 min, 25%A; 61-75 min, 95%A; 76-80 min, 95% A, were appropriate in the analysis. An autosampler system was used for sample injections (20 µl) and flow rate was 0.8mL min^{-1}. Minimum re-equilibrium time between two injections was 15 min and each sample was analyzed twice from the same vial.

High performance liquid chromatography coupled with mass spectrometry (HPLC-MS): HPLC-MS was performed with an 1100 Series HPLC and quadrupole ion trap mass spectrometer (ThermoFinnigan LCQ-DECAXPlus). The HPLC conditions were same as above mentioned. The mass spectra were recorded using quadrupole ion trap mass spectrometer with sample ionized by an electro spray ionization (ESI) source operated in negative mode and using vaporizer temperature 550°C, sheath and auxiliary nitrogen flow pressures of 30 and -10 psi, respectively. Capillary temperature 350°C and capillary voltage -15°C were optimum in this analysis. The mass spectrometer was controlled by Xcalibur 1.3 software (ThermoFinnigan) and programmed to record survey scans in the range m/z 200-2000, in TIC mode. The recorded data were analyzed and identified by comparison retention times, UV spectra and TIC patterns in MS with standards and cited literature. The peak area variation of those compounds were calculated which were common in at least two species. The obtained data were formulated in form of a matrix and used to construct phenogram by using MVSP software to indicate infrageneric position of different taxa of *Clematis* genus (Fig. 2).

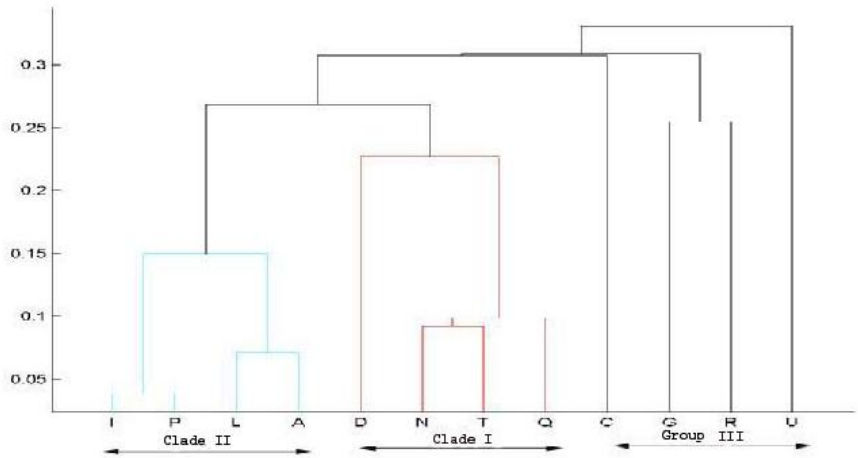

Fig. 2. Phenogram showing affinity relationships among taxa of *Clematis* genus based on chemotypic characters, as determined by Euclidian distance, average link (Classical "Unweighted Pair-Group Method Using Arithmetic Averages") algorithm. The alphabetical names are same as presented in Table 1.

Results and Discussion

Measurement of Morphological characters: Morphologically important characters were weighed in terms of numerical values and formulated in form a matrix. The obtained data from various studied specimens was tabulated as in (Table 2) and used for phenogram construction by using MVSP software (Fig. 1).

Identification of saponins compounds: To identify saponin in different extracts of taxa of *Clematis*, their HPLC retention times (RT), ultraviolet spectra (UV) and ESI mass spectra were compared with those of standards previously isolated from *Clematis ganpiniana* (Sun *et al.*, 2007) or with saponins present in the genus which had been identified (Shao *et al*., 1995; Chirva *et al*., 1974; Shao *et al*., 1996; Kizu *et al*., 1995; Kawata *et al.*, 1998; Song *et al.*, 1992) as in Table 3. The RTs, PAs (peak areas), UV spectra and ESI mass spectra of peaks of different compounds present in analyzed samples were measured. The data in binary form (presence/absence) of different compounds and their PAs were formulated in form of matrix and used for cluster formation (Table 4). Only five standards of saponins were available for chromatographic comparison (Huzhangoside B, Clematichinenoside C, Seiboldianoside A, Huzhangoside D, Clematichinenoside B), so that other saponins detected in the analysis could not be identified by HPLC. No standard compounds were available for the compounds 6-9, but their RT, UV spectra and MS spectra from ESI source were compared with previously identified compounds in the genus and four compounds were tentatively found to be Clemochinenoside A, Songaroside B, Clemastanoside A, Clematichinenoside C (isomer) with molecular weight 684.6, 1028.5, 1378.6, and 1499 respectively, by LC–ESI-MS (Table 3).

Presence and distribution of saponins in genus *Clematis*: This distribution pattern of saponins mirrors taxonomic relationships among the taxa and predict their ecological and morphological characteristics. The distribution of saponin profile of studied species of genus *Clematis* is given in (Table 4). Among the identified compounds; Huzhangoside D showed high concentration in *C. chinensis, C. henryi, C. armandii* and *C. terniflora* (Fig. 3) and, Huzhangoside B showed high quantity in *C. chinensis, C. huchouensis, C. finetiana* and *C. peterae*. Clemochinenoside A depicted high amount in *C. heracleifolia* and *C. armandii* and, other compounds were found moderate to minor amounts or some times as traces in different analysed species (Table 4).

First group (clade I) of CCT includes species of subsect. *Rectae* which share all five standard compounds but variable in quantitative measures and are fairly aggregated as one clade branch of CCT (Fig. 4). The compound 3 (SDA) seems to be the mostly restricted with moderate quantity to clade II (D, N, Q, T) making it special chemo-marker for identification and classification. However, distinctive characteristics of subsect. *Clematis* are the presence of compounds HGD, SDA, CCC in large to minor quantity. Furthermore, compounds CCB, SDA, CCC, HGB, CCCI, CSS, SSB can be helpful in demarcating taxa boundaries at subgenus level in the genus *Clematis*. Among these, *C. huchouensis* (U) sample possessed these saponin compounds (HGD, SDA, CCC, HGB) and appeared as one line in CCT, *C. heracleifolia* (G) consisted of compounds (HGD, CCA, CSS) and *C. intricata* (C) had compounds (HGB, HGD) but latter one (HGD) in trace. The species *C. henryi* (R) sect. *Connatae* (subgen. *Viorna*) has compounds (HGD, CCA) common with other species *C. heracleifolia* (G) and appears sister clade branch with species G in third group (III) of CCT.

Table 3. HPLC Retention times, UV absorption maxima, and Molecular weight [MH^+(m/z)].

S. No.	Compound name (abbreviations)	R_t (min) HPLC/UV	$U\overline{\lambda}_{max}$ (nm)	MH^+ m/z)	References
1.	Clematichinenoside B (CCB)	44.70	206	1514	[Shao1995]
2.	Huzhangoside D (HGD)	46.94	208	1352	[Kizu1995]
3.	Seiboldianoside A (SDA)	47.77	206	1352	[Kizu1995]
4.	Clematichinenoside C (CCC)	51.20	208	1498	[Shao1996]
5.	Huzhangoside B (HGB)	54.35	208	1336	[Kizu1995]
6.	Clemochinenoside A (CCA)	18.47	206	684.6	[Song1992]
7.	Songaroside B (SSB)	55.60	208	1028.5	[Chirva1974]
8.	Clemastanoside A (CCA)	20.6	206	1378.6	[Kizu1995]
9.	Clematichinenoside C (isomer) (CCCI)	42.6	206	1499	[Kawata1998]

Table 4. HPLC Retention times, UV absorption maxima, and Molecular weight [MH^+(m/z)].

S. No.	Retention time (Min)	Chemical constituents (abbreviations)	Percentage composition (%)
1.	18.47	Clemochinenoside A (CCA)	0.60 ± 0.105
2.	20.60	Clemastanoside A (CCA)	0.33 ± 0.115
3.	42.60	Clematichinenoside C (isomer) (CCCI)	8.26 ± 0.362
4.	44.70	Clematichinenoside B (CCB)	5.40 ± 0.272
5.	46.94	Huzhangoside D (HGD)	49.6 ± 0.926
6.	47.77	Seiboldianoside A (SDA)	27.8 ± 0.681
7.	51.20	Clematichinenoside C (CCC)	1.26 ± 0.217
8.	54.35	Huzhangoside B (HGB)	2.33 ± 0.25
9.	55.60	Songaroside B (SSB)	4.60 ± 0.284

Table 5. Saponin distribution in the *Clematis* genus using HPLC-UV & ESI-MS.

S. No.	Species code	CCB	HGD	SDA	CCC	HGB	CCA	CCCI	CSS	SSB
1.	U	--	+++	--	--	Tr	--	--	--	--
2.	G.	--	++	--	--	--	+++	--	+	--
3.	N	+	+++	++	+	+++	+	+	+	--
4.	L	+	++	Tr	+	++	+	--	--	--
5.	I	+	++	Tr	+	+	++	+	+	+
6.	R	--	+++	--	--	--	++	--	--	--
7.	D	+	++	++	+	+++	+	++	+	--
8.	Q	+	+++	++	+	++	+	+	+	--
9.	C	--	Tr	--	--	++	--	--	--	--
10.	A	tr	++	+	+	+++	+	+	--	--
11.	T	++	+++	--	+	++	--	--	--	--
12.	P	++	++	Tr	+++	+	tr	--	--	+

Abbreviations used above: CCB: Clematichinoside B; HGB: Huzhangoside D; SDA: Sieboldianoside A; CCC: Clematichinoside; HGB: Huzhangoside B; CCA: Clematichinenoside A; CCCI: Clematichinoside Isomer; CSS: Clemastanoside; SSB: Songaroside B; +++: large concentration; ++: large to moderate concentration; +: minor concentration; tr: trace concentration; --: not detectable.

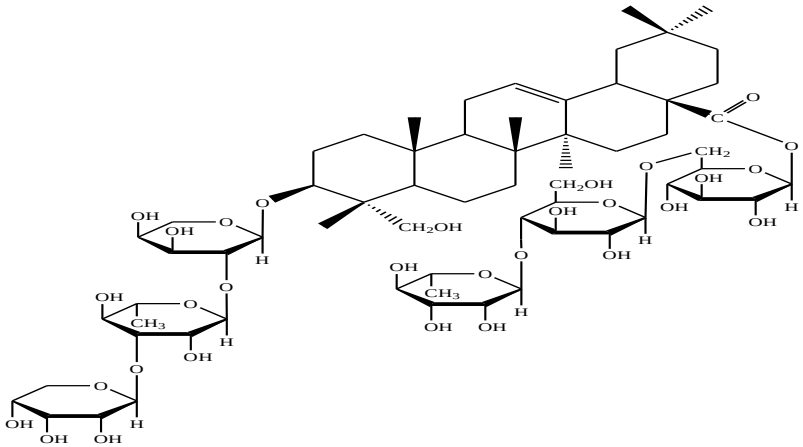

Fig. 3. Huzhangoside D.

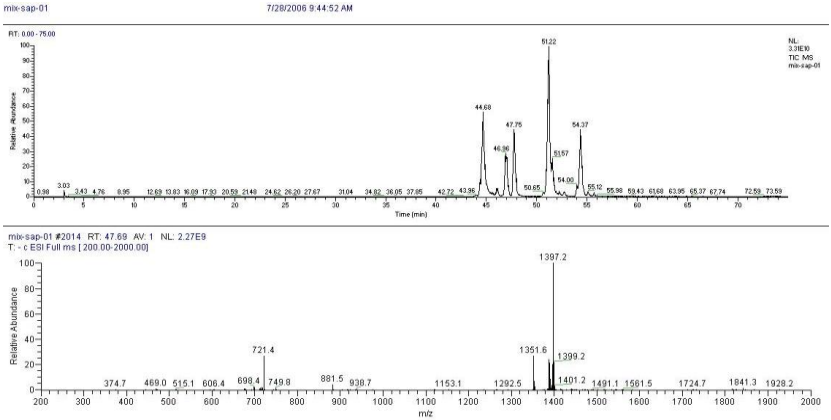

Fig. 4. ESI-MS spectrum of standards showing SDS compound with MW 1352.

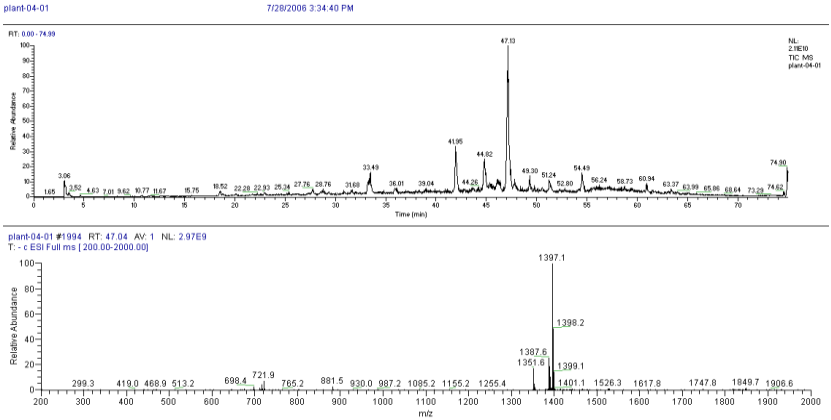

Fig. 5 . ESI-MS spectrum showing presence of different saponins, SDS with MW 1352.

A matrix was constructed based on data of presence or absence of saponin compounds and their PAs variation present in different taxa. A chemical cluster tree (CCT) was generated from this matrix data using Euclidian distance, average link (Classical "Unweighted Pair-Group Method Using Arithmetic Averages") algorithm by MVSP software (Fig. 2). According to chemical profile of saponins present in analyzed taxa of the genus, there are three main groups in CCT (Fig. 2). The clade I (subsect. *Clematis*) consists of *Clematis apiifolia* (L), *C. argentilucida* (I), *C. ganpiniana* (P), and *C. peterae* (A) species which are morphologically so similar that it is hard to identify them merely by classical method (Wang, 1998) but if numerical based approach is used it can well distinguish taxa from each other but still it there are few discrepancies at distal level. In chemical analysis these taxa appeared as one aggregate in CCT which is broadly consistent with numerical based cluster, however individual species are better separated due to qualitative or quantitative variation of compounds (Fig. 2) and these findings support previous classification system (Tamura, 1987). Second group (clade II) of CCT comprises of *Clematis finetiana* (D), *C. armandii* (Q), *C. chinensis* (N) and *C. terniflora* (T) belongs to subsect. *Rectae*, albeit very closely related on morphological grounds yet well isolated in this chemical analysis. The clade II species contained all five standard compounds and aggregated in one clade branch of CCT but still they are well separated at species level due to quality or quantity variation of different compounds. The compound 3 (SDA) is chemotaxonomic marker of this subsect (Fig. 5). Third aggregate (group III) contains those species which belong to different sections; *C. heracleifolia* (sect. *Tubulosae*), *C. huchouensis* (sect. *Viticella*), *C. intricata* (sect. *Meclatis*) and *C. henryi* (sect. *Connatae*) are fairly separated from each other as well as from other two most similar clades (clade I and II) in MCT that broadly corroborate with previous classification. In case of CCT, group III although well separated from other two clades (I & II) but intra-group it has differential occurrence of taxa such as species G and R belong to one line and secondly species U genetically appears at far distant from others. The close affinity between species R and G is substantially different from previous classification systems (Wang, 2005; Jonathan, 2004). Among analyzed taxa, species D of subsect. *Rectae* has more close-ness with taxa of subsect. *Clematis* than to other allies (Fig. 2).

The numeric based results congruently favours chemical based classification, however some minor discrepancies within group III of CCT is inviting for further taxonomic exploration. This may be due to analytical error or small number of samples analyzed. It is also plausible that these species may be genetically more related to each other. Hence, further detailed and comprehensive taxonomic studies based on chemical analysis are inevitable to solve this plethora and others, for definite identification and classification of many species of genus *Clematis* (Wang, 1998).

Concluding perspectives

The classification of different Chinese *Clematis* species by chemotypic and numeric taxonomical approaches well differentiated infrageneric relationships. It was seen that saponin profile of genus *Clematis* taxa ubiquitously can resolve the discrepancies hitherto present in the genus (Wang, 1998). Moreover, further detailed morphological and chemotaxonomic analysis throughout the whole range of distribution of *Clematis* taxa may be helpful to study the comprehensive phylogenetic and taxonomic position of this large and complex genus.

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