MOLECULAR SYSTEMATICS OF THE TRIBE CIMICIFUGEAE AND ALLIED GENERA IN THE RANUNCULACEAE.

by

IKUE HASEGAWA

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Nov. 18, 1992
Date

Co-chairmen of Examining Committee
Dr. Dennis W. Stevenson
The New York Botanical Garden

Nov. 18, 1992
Date

Co-chairmen of Examining Committee
Dr. Tetsuo Koyama
The City University of New York

Dec. 1, 1992
Date

Executive officer

Dr. P. Mick Richardson
The Missouri Botanical Garden

Dr. Michael Balick
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The City University of New York

Dr. Olle Pellmyr
The University of Cincinnati

Dr. Mikio Ono
Off-campus Reader
The Tokyo Metropolitan University

Dr. Tatemi Shimizu
Off-campus Reader
The Kanazawa University

Supervisory Committe

The City University of New York

Abstract

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by

Ikue Hasegawa

Co-advisers: Dr. Dennis Stevenson
Dr. Tetsuo Koyama

The Phylogenetic analysis of the tribe cimicifugeae including three monotypic Asian genera (Anemonopsis, Beesia, and Souliea) and allied genera in the Ranunculaceae inferred from *rbc*L sequences was conducted for the first time.

The Ranunculaceae Juss. is a large and complex family, widely distributed, especially in the temperate and boreal regions of the northern hemisphere (Cronquist, 1981). Many species of Ranunculaceae are commonly used for horticultural and medicinal purposes and thus one might expect this large family to be a thoroughly studied and understood group of plants. However, there have been different taxonomic views among taxonomists regarding

the classification of Ranunculaceae. These differing taxonomic views are caused in part by the lack of data on this archaic and heterogenous group of plants, which are morphologically simple, but highly variable. Therefore, it is difficult to find good conservative morphological characters and the group is in great need of detailed systematic re-examination employing modern taxonomic methods. This study is to obtain additional characters (*rbc*L sequence) for resolving incongruencies between phylogenies generated in previous morphological, cytological and serological studies and to perform phylogenetic analyses to provide a comprehensive and broadly based assessment of evolutionary relationships of the Cimicifugeae.

1333 bp nucleotide sequences of the *rbc*L gene were obtained for 15 genera of the Ranunculaceae and two outgroup families. A total of 247 variable nucleotide positions were identified, 120 of which were phylogenetically informative. Phylogenetic analyses of the rbcL data generated three equally parsimonious trees with a consistency index of 0.641. The clade that correspond to the tribe Cimicifugeae was well supported and showed a new alignment of Beesia and Anemonopsis as a sister group. The tradisional view of Actaea and Cimicifuga as a monophyletic taxa is not shown when an Asian monotypic genus, Souliea is included in a study. A new alignment is Souliea is closer than Actaea to Cimicifuga. The other genera in this study were congruent with chromosome information with respect to the tribal circumscription in the Ranunculaceae.

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TABLE OF CONTENTS

Table of contents continued

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER I. INTRODUCTION

The Ranunculaceae Juss. is a large and complex family consisting of approximately 66 genera and 2000 species (Takhtajan, 1987), widely distributed, especially in the temperate and boreal regions of the northern hemisphere (Cronquist, 1981). The recent geographical analysis by Ziman and Keener (1989) regards the Ranunculaceae as an ancient temperate family that originated in the Cretaceous within montane temperate floras of the Northern Hemisphere. Most authors (Cronquist, 1981; Takhtajan, 1987; Thorne, 1976) regard the Ranunculaceae as monophyletic, retaining primitive or less specialized characters among the herbaceous angiosperms. However, the family is quite heterogenous, and its monophyly has since been questioned. The primitiveness of the family has been fully established by workers in comparative morphology and floral anatomy (Kumazawa, 1932a, b; Tamura, 1966). Some of the primitive characters found in the family are: numerous and spiral arrangement of floral parts, apocarpy, imperfect carpel closure, and foliclular fruit. Peculiar traits are some anatomical features not commonly found in dicots, such as fused cotyledons, trimerous flowers, V-shaped xylem, and development of pollen grains and endosperm more characteristic of the monocots than of the dicots (Nowicke and Skvarla, 1981). Several advanced

characters are also common, such as finely dissected leaves, vessels with simple perforations, racemose inflorescences, unisexual and zygomorphic flowers, specialized spurred petals, syncarpy (with each carpel still distinct), and achenes.

Many species of Ranunculaceae are commonly used for horticultural and medicinal purposes and thus one might expect this large family to be a thoroughly studied and understood group of plants. Nevertheless, the generic distinctions within the family are admittedly weak and the phylogenetic relationships among genera are still unclear. Two problems contribute to this confusion. First, despite the availability of information on numerous characters (as mentioned above), the present phylogenetic reconstructions for this family are based on a single character or a group of characters (Tamura, 1962), for example, follicle versus achene, or chromosome number and size. Second, many monotypic or ditypic, primitive genera included in the tribe Cimicifugeae by several authors (Janchen, 1949 and Hutchinson, 1973), such as Anemonopsis (a monotypic genus distributed in the temperate zone of Japan), Beesia (two species in temperate western China), Souliea (a monotypic genus distributed in China), are very important for understanding the phylogeny of the family as a whole, and the information which these genera provide might well help to establish linkage among certain groups. They are however hard to obtain for study and often overlooked. Many of the monotypic genera are endemic to eastern

Asia in localities where botanists still do not have easy access. As a result, there has been wide disagreement among taxonomists regarding the classification of Ranunculaceae. These differing taxonomic views are caused in part by the lack of data on this archaic and heterogeneous group of plants, which are morphologically simple, but highly variable. The group is in great need of detailed systematic revision employing modern taxonomic methods.

The goals of this study are to use the available information to resolve this debate by examining rbcL sequences of genera in the tribe Cimicifugeae and its allies to obtain additional characters for resolving incongruencies between phylogenies generated in previous morphological, cytological and serological studies; and by perform phylogenetic analyses of data sets of DNA and macromorphology to provide a comprehensive and broadly based assessment of evolutionary relationships of the Cimicifugeae.

This study should provide a better understanding of the systematic relationships of ancestral genera in the tribe Cimicifugeae and also provide a basis for understanding the early evolution of the Ranunculaceae.

CHAPTER II. A BRIEF TAXONOMIC HISTORY OF THE TRIBE CIMICIFUGEAE

The Ranunculaceae, as a natural family of plants, have been recognized for over two hundred years (A. L. de Jussieu, 1789). The taxonomic history of Ranunculaceae has been presented in detail by Gregory (1941) and Tamura (1966). Outlines of major classifications are shown in Table 1.

Cimicifugeae has historically been treated either as a tribe or subtribe. Previous views are summarized as follows:

1. The tribe Cimicifugeae established by Gray (1848) was composed of Actaea, Cimicifuga, and Hydrastis, as the fifth tribe of the Ranunculaceae.
2. Lindley (1853) added Trautvetteria, Xanthorhiza, and Podophyllum, to the Cimicifugeae Gray, and invalidly changed the tribal name to Actaeae.
3. Bentham and Hooker (1862) included only Actaea and Cimicifuga in a subtribe Cimicifugeae of the tribe Cimicifugeae.
4. Anemonopsis was *incerta cedis* until Baillon (1868) placed it in the tribe Aquilegieae with Aquilegia, Xanthorhiza, Helleborus, Trollius, etc.. Actaea was regarded to be near Thalictrum, Clematis, etc. and included in the tribe Clematideae.

5. Based on histological studies, Meyer (1884) and Marié (1885) concluded that Actaea and Cimicifuga are closer to Thalictrum in their stem anatomy, and Anemonopsis is anatomically more similar to Actaea.

6. Prantl (1888) divided the family into three tribes based on fruit types. He transferred the genera placed by earlier authors in the tribe Cimicifugeae into the tribe Helleboreae, characterized by follicular fruits with many seeds.

7. Delpino (1899), who emphasized the systematic importance of characters of the nectary and the inflorescence, grouped the genera Cimicifuga, Actaea, Xanthorhiza, Coptis, Isopyrum and Hydrastis together in the tribe Cimicifugeae.

8. Schrödinger (1909) recognized two essential types of the petal, i.e. 'becherig' (beak-like) and 'flächig' (shallow), and concluded that Actaea, Cimicifuga, and Anemonopsis are most closely related to each other, and less so to Coptis and Xanthorhiza. Consequently he placed these five genera together in the tribe Cimicifuginae.

9. Lotsy (1911) raised the rank of Prantl's tribes to subfamilies and further divided them into tribes and subtribes. He placed Anemonopsis, Cimicifuga, Actaea, Coptis, and Xanthorhiza in the subtribe Cimicifuginae.

10. Langlet (1932) and Gregory (1941) divided the family into tribes based on chromosome morphology and number. The genera of the

tribe Cimicifugeae sensu Gray are placed either in the subtribe Cimicifuginae or in the tribe Helleboreae.

11. The two Chinese endemic genera Beesia and Souliea are often not included in taxonomical studies. However, when they are included in a study, they are usually placed in the tribe Cimicifugeae. Tamura (1966) removed Beesia to the tribe Trollieae and retained four genera, Actaea, Cimicifuga, Anemonopsis, and Souliea in the tribe Cimicifugeae. In the most recent classification by Tamura (1990, 1991, 1992), Beesia has removed from the tribe Trollieae and place it in its own subtribe Beesiinae.

Although a new classification of Ranunculaceae has proposed by Tamura (1990, 1991, 1992), in this study, I will use Tamura's old system (1966, 1968) which currently in the most widely accepted and used. The outline of his old classification is shown in Table 2.

Table 1. Outlines of major classification.

De Candolle (1824)	Bentham & Hooker (1862)	Prantl (1888)
Tribe I. Clematideae: Clematis, Naravelia	Tribe I. Clematideae: Clematis, Naravelia	Tribe I. Paeonieae: Glaucidium, Hydrastis, Paeonia
Tribe II. Anemoneae: Thalictrum, Tetractis, Anemone, Hepatica, Hydrastis, Knowltonia, Adonis	Tribe II. Anemoneae: Thalictrum, Anemone, Knowltonia, Adonis, Callianthemum, Myosurus	Tribe II. Helleboreae: Caltha, Trollius, Nigella, Callianthemum, Eranthis, Leptopyrum, Isopyrum, Coptis, Xanthorhiza, Anemonopsis, Actaea, Aquilegia, Delphinium, Aconitum
Tribe III. Ranunculeae: Myosurus, Ceratocephalus, Ranunculus, Ficaria	Tribe III. Ranunculeae: Trautvetteria, Ranunculus, Hamadryas, Oxygraphis	Tribe III. Anemoneae: Anemone, Clematis, Myosurus, Oxygraphis, Trautvetteria, Ranunculus, Thalictrum, ?Hamadryas, Adonis
Tribe IV. Helleboreae: Caltha, Trollius, Eranthis, Helleborus, Coptis, Isopyrum, ?Enemion, Garidella, Nigella, Aquilegia, Delphinium, Aconitum	Tribe IV. Helleboreae: Subtribe Caltheae: Caltha, Calathodes, Glaucidium, Hydrastis, Trollius, Helleborus, Eranthis Subtribe Isopyreae: Coptis, Isopyrum, Nigella, Aquilegia, ?Anemonopsis Subtribe Delphinieae: Delphinium, Aconitum Subtribe Cimicifugeae: Actaea, Cimicifuga, Xanthorhiza	
Tribe V. ?Paeoniaceae: (Ranunculaceae spuriae) Actaea, Xanthorhiza, Paeonia	Tribe V. Paeonieae: Paeonia	

Table 2. Classification of Tamura (1966, 1968), containing 6 subfamilies and 10 tribes.

Subfamily Ranunculoideae:
- Tribe Anemoneae:
  - Subtribe Anemoninae:
    - Anemone
    - Hepatica
    - Knowltonia
    - Pulsatilla
  - Subtribe Clematidinae:
    - Clematis
    - Naravelia
  - Subtribe Kingdoniinae:
    - Kingdonia
- Tribe Ranunculeae:
  - Subtribe Ranunculinae:
    - Ranunculus
    - Myosurus
  - Subtribe Trautvetteriinae:
    - Trautvetteria
  - Subtribe Adonidinae:
    - Adonis

Subfamily Helleboroideae:
- Tribe Cimicifugeae:
  - Cimicifuga
  - Actaea
  - Anemonopsis
  - Souliea
- Tribe Helleborinae:
  - Subtribe Helleborinae:
    - Helleborus
    - Eranthis
  - Subtribe Nigellinae:
    - Nigella

Table 2. continued.

Tribe Trollieae:
Trollius
Caltha
Beesia

Tribe Delphineae:
Delphinium
Consolida
Aconitum

Subfamily Thalictroideae:
Tribe Thalictreae:
Thalictrum

Subfamily Isopyroideae:
Tribe Isopyreae:
Subtribe Isopyrinae:
Isopyrum
Subtribe Aquilegiinae:
Aquilegia

Subfamily Coptidoideae:
Tribe Coptideae:
Coptis
Xanthorhiza

Subfamily Hydrastidoideae:
Tribe Hydrastideae:
Hydrastis

CHAPTER III. REVIEW OF MORPHOLOGICAL AND ANATOMICAL STUDIES

General Morphology

Morphology is traditionallythe most fundamental information on which all taxonomy rests. In the following, morphological features considered important in previous classification of Ranunculaceae are briefly described.

General habit -- Most members of Ranunculaceae are perennial herbs, but annual or biennial herbs are occasionally found. Annual or biennial habit is one of the important characters which delimit these groups from allied ones. The woody habit, generally regarded as a primitive feature in angiosperms, is also rare in this family, where it is considered as a secondary condition.

Flowers -- Flowers are usually actinomorphous, but zygomorphy (certainly an advanced character) is found in Delphinium, Consolida, and Aconitum. In the Ranunculaceae, the hermaphoditic flower is common; but the unisexual flower is not rare and is found in Cimicifuga, Coptis, Clematis, and Thalictrum. Since the flowers of the Ranunculaceae are so variable, they are not as informative a character as found in other families. However, they show some developmental series derived from a fundamental type that help in understanding the family's evolution.

Inflorescences -- The majority of the Ranunculaceae have the shoot and its branches terminated by a flower, so the inflorescence is generally determinate. Indeterminate inflorecences are found in Aconitum, Delphinium, Cimicifuga, Actaea, Souliea, Consolida, etc. There are several inflorescence patterns that seem to be applicable in the family. When the axillary cymes have a single flower, the inflorescence is a true corymbiform cyme and this type is seen in Caltha, Helleborus, Anemonopsis, Aconitum, Delphinium, Aquilegia, Ranunculus, etc. The racemiform inflorescence is observed in Cimicifuga, Actaea, Souliea, Thalictrum, Coptis, etc.

Leaves -- The leaves are in rosettes or alternate, but opposite in Clematis. They are commonly much-divided or palmately lobed, but a wide range of shapes are found within the family. For examples, entire and narrow in Myosurus and some species of Ranunculus, cordate in some Caltha species and Beesia, and pinnately lobed in Xanthorhiza and Clematis. The petioles are broadened into a sheathing base. Stipules are absent, except in Thalictrum, Caltha, Trollius, and Ranunculus.

Anatomy

In some groups of the Ranunculaceae, the petiolar anatomical feature is constant and useful as a taxonomic character. According to Tamura (1964), presence or absence of ventral bundles or medullary bundles, the rough number of main and small bundles,

dominance of distal bundles in a bundle arc, absence or presence of bundle sheath, etc. are important.

The Stem —— In the aerial stem, vascular bundles are usually provided with the bundle sheath. Kumazawa (1932b) recognized two types: one is the cap or the arc of fibrous cell groups present immediately outside of the phloem (Anemone or A-type) and the other surrounds the bundle (Ranunculus or R-type). A-type is observed in the petioles of Trollius, Anemonopsis, Actaea, Thalictrum, etc. and R-type is distinct in Xanthorhiza and Coptis. In the cross section of the stem, vascular bundles are arranged not in a perfect circle, but more or less irregularly. Well developed leaf-sheath surrounding the stem is found in many genera, such as Cimicifuga, Actaea, Anemonopsis, Thalictrum, Adonis, etc..

The Petioles —— The petioles were studied by Worsdell (1908), Schrödinger (1914), Troll (1932), and Tamura (1964). In some groups of the Ranunculaceae, the anatomical feature of the petiole is constant and considered a taxonomically significant character by Tamura (1964, 1966). Furthermore, an important fact is that the petiolar anatomy suggests the evolutionary course of the species or groups in showing the series of reduction of a ventral bundle or of medullary bundles. The anatomical features of the stem, such as V-shaped xylem and irregular arrangement of bundles, are generally observed also in the petiole.

Palynology

Pollen morphology has proved to be of great value in interpreting problems related to the origin, migration, and evolution of floras (Radford, 1986). Earlier workers such Kumazawa (1936), Wodehouse (1936), and Ikuse (1956) described and illustrated the pollen grains of the Ranunculaceae. The pollen morphology of many Ranunculaceae is illustrated in Meacham (1981) and in the work of Nowicke & Skvarla (1982). Meacham showed that the pollen grains of the family are tricolpate or tricolpate-derived (hexacolpate to polyforate, with many intermediates). Pollen characters such as type of aperture and aperture number, sculpturing pattern, provide data useful in a phylogenetic analysis. From the SEM photographs published in Meacham, and Nowicke & Skvarla, I determined the characters mentioned above for the genera included in this study. The descriptions for the species in this study are listed in Table 3. Meacham (1981) suggested that pollen morphology seems to show that the Ranunculaceae is somewhat isolated from the other families surveyed (Lardizabalaceae and Berberidaceae) and contains several distinct evolutionary lines. He mentioned an example of Gregory's (1941) Thalictreae to be one such line which has a fairly uniform leaf morphology. A survey of leaf morphology might be useful in supporting groups recognized on the basis of pollen morphology.

Embryology

A wide array of embryological characters, more than 50 in general for each taxon of angiosperms (Tobe, 1989), have been applied to systematic and evolutionary studies and have clarifed the position of certain problematic families or genera. For example, Thymelaeaceae, which have most often been assigned to Myrtales (e.g., Cronquist, 1981), clearly differ from the order by their embryological characters and can be safely excluded from the order (Tobe and Raven, 1983). Despite its evident systematic value, and the fact that the embryogenesis of the Ranunculaceae is fairly well studied, embryological characters are not commonly used in phylogenetic analyses of the Ranunculaceae due to their lack of variability and irregularity within the family. Johansen (1950) considered Myosurus minimus as the most representative of the Ranunculaceae. In M. minimus the four-celled proembryo is developed as in the typical Onagrad type (a vertical wall in the apical cell and a transverse wall in the basal cell give rise to a T-shaped tetrad), so the Ranunculaceae is known to have the Onagrad-type of embryogeny and named the Myosurus-variation of Onagrad type.. Studies of embryology in the Ranunculaceae were reviewed for inclusion in phylogenetic analyses. The embryological characters are summarized in Table 4.

Table 3. Pollen characters.

	Number of aperture	Type of aperture	Sculpturing pattern
Actaea	3	colpate	echinate
Adonis	3	colpate	echinate
Anemonopsis	3	colpate	perforate
Beesia	3	colpate	echinate
Caltha	3	colpate	echinate
Cimicifuga	3	colpate	perforate-echinate
Clematis	3	colpate	echinate
Coptis	~ 8	polycolpate	echinate
Eranthis	3	colpate	echinate
Isopyrum	3	colpate	echinate
Souliea	0	no aperture	minutely echinate
Thalictrum	~ 8	polycolpate	echinate
Trollius	3	colpate	striate
Xanthorhiza	~ 8	polycolpate	echinate
Hydrastis	3	colpate	striate
Caulophyllum *	3	colpate	echinate

* outgroup.

selected reference: Marcheam, 1981; Erdtman, 1952; Nowicke & Skvarla, 1981, 1982.

Table 4. Embryological characters.

	position of ovule	integument number	nature of nucellus	development of embryo sac
Actaea[1)]	Anatropus	bitegmic	crassinucellar	polygonum
Anemonopsis[2)]	Anatropus	bitegmic	tenui-, or crassinucellar	polygonum
Cimicifuga[3)]	Anatropus	bitegmic	tenui-, or crassinucellar	polygonum
Caltha[4)]	Anatropus	bitegmic	crassinucellar	polygonum
Trolius[5)]	Anatropus	bitegmic	tenui-, or crassinucellar	Allium or polygonum
Adonis[6)]	Anatropus	bitegmic	tenui-, or crassinucellar	Allium
Clematis[7)]	Anatropus	unitegmic	pseudo-crassinucellar	polygonum
Thalictrum[8)]	Anatropus	bitegmic	crassinucellar	polygonum
Eranthis[9)]	Anatropus	bitegmic	crassinucellar	polygonum
Hydrastis[10)]	Anatropus	bitegmic	crassinucellar	polygonum

Selected references:

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CHAPTER IV. CYTOLOGY

Previous classifications of the Ranunculaceae were traditionally based on morphological characters; these were significantly altered after cytological studies.

Among the early works on cytology, Langlet (1932) made the most complete and significant contribution to the systematics and phylogeny of the family. When Langelet (1927) began publishing chromosome counts, he noticed that the chromosomes fell into two sharply and markedly different groups based on both size and "type". One type he described as predominantly large, always long and bent and named it the Ranunculus type (R-type). The second type he described as small and kidney-shaped and named it the Thalictrum type (T-type). Langlet (1932) divided the Ranunculaceae into two subfamilies and eight tribes (shown in Table 5) based on these two types of chromosomes. Gregory (1941) proposed a phylogeny of Ranunculaceae based mainly on the chromosomes. He accepted Langlet's subfamilies but recognized only the six tribes shown in Table 6 . The genera included in the present study are placed in tribe Helleboreae by both authors based on the large R-type chromosomes with a base number of X=8 .

The chromosome number and karyotypes are well presented in a series works of Kurita (1957 - 1967). Chromosome data for one of the Chinese endemics, Beesia calthifolia, was published by Shang in

1985. The chromosome number and karyotype of the other Chinese monotypic genus Souliea vaginata, is presented herein (Hasegawa and Feng, 1991).

Living plants of Souliea vaginata Franch. were collected from Maerkang, Sichuan Province at 3700 m altitude and brought back to Kanazawa, Japan for cytological study.

Preparations for observation of mitotic phases were carried out as follows: the root tips were pretreated with 0.002M 8-hydroxyquinoline aqueous solution for 1.5-3 hours at 18-20°C and fixed in the 2:1:1 mixture of absolute ethanol, chloroform and acetic acid at 4°C for more than 12 hours (usually overnight). The root tips were treated with 45 % acetic acid at 0°C (ice water) for 15 minutes and macerated in 1N HCL for 20 to 30 seconds at 60°C, then stained with 1% aceto-orcein for 45 to 60 minutes before they were squashed. Chromosome morphology is usually studied at the metaphase stage of mitosis, when chromosomes are contracted to the maximum amount and they are most easily stained. Cytological examination and photographs were made with a Nikon microscope. Voucher specimens (Peng Feng and Tang Shijie 91001) are preserved in the herbaria of Nanjing Botanical Garden (NSM) and Kanazawa University (KANA).

Result

Souliea vaginata has a somatic chromosome number of 2n=16 which is R-type (Figure 1) and chromosome lengths are shown in Table 6. The chromosome complement can be subdivided into four major groups (Figure 2): 1) two pairs of V-shaped median chromosomes; 2) three pairs of V-shaped submedian chromosomes of which the second pair has a clear secondary constriction at the distal portion of the short arm (Fig. 2. D); 3) two pairs of J-shaped chromosomes with an arm ratio of about 1:2; and 4) one pair of subterminal chromosomes, of which the short arms are very minute and possess a characteristic small satellite (Fig. 2. H).

Table 5. Classification of Ranunculaceae based on the types of chromosomes (Langlet, 1932).

Subfamily Thalictroideae (T-type)

Tribe I. Thalictreae

Subtribe Isopyrinae:

Isopyrum, Leptopyrum, Aquilegia

Subtribe Thalictrinae:

Thalictrum, Anemonella

Tribe II. Coptideae:

Coptis, Xanthorhiza

Subfamily Ranunculoideae (R-type)

Tribe III. Helleboreae

Subtribe Helleborinae:

Helleborus, Eranthis

Subtribe Cimicifuginae:

Anemonopsis, Cimicifuga, Actaea

Tribe IV. Ranunculeae:

Myosurus, Trautvetteria, Ranunculus

Table 5. continued.

Tribe V. Anemoneae

Subtribe Anemoninae:

Anemone, Knowltonia

Subtribe Clematidinae:

Clematis

Tribe VI. Trollieae

Subtribe Trolliinae:

Caltha, Trollius

Subtribe Delphiniinae:

Delphinium, Aconitum

Tribe VII. Adonieae:

Callianthemum, Adonis

Tribe VIII. Nigelleae:

Nigella, Komaroffia

Table 6.

Classification of the Ranunculaceae based mainly on chromosomes (Gregory, 1941).

A. Large Ranunculus-type (?) chromosomes. Basic number = 5

Tribe I. Paeonieae

1. Paeonia

B. Large Ranunculus-type chromosomes. Basic number = 8

a. Fruit follicle, capsule, or berry

Tribe II. Helleboreae

2. Caltha
3. Trollius
4. Helleborus
5. Eranthis
6. Nigella
7. Actaea
8. Cimicifuga
9. Anemonopsis
10. Delphinium
11. Aconitum

Table 6. continued.

b. Fruit an achene, usually one-seeded

Tribe III. Anemoneae

12. Anemone

13. Clematis

14. Ranunculus

15. Callianthemum

16. Adonis

C. Small Thalictrum-type chromosomes. Basic number = 7

Tribe IV. Thalictreae

i. Fruit follicular, many seeded

17. Aquilegia

18. Isopyrum

ii. Fruit an achene, one seeded

19. Anemonella

20. Thalictrum

D. Small slender (Coptis-type) chromosomes. Basic number = 9

Tribe V. Coptideae

i. Fruit several seeded

21. Coptis

ii. Fruit two seeded

22. Xanthorhiza

E. (Not examined by Gregory)

Tribe VI. Hydrastideae (position uncertain)

23. Hydrastis

Figure 1. Microphotograph of somatic chromosomes at metaphse of Souliea vaginata (2n = 16).


10µm
1

Figure 2. Chromosome complement of Souliea vaginata.

A
B
C
D
E
F
G
H
10μm
2

Table 7. Measurements of the chromosomes of Souliea vaginata in µm.

Chromosome	Length of arms	Total	Index
A	5.4+5.6	10.0	0.85
	5.7+4.6	10.3	0.81
B	5.4+4.2	9.6	0.78
	5.4+3.1	8.5	0.57
C	4.3+4.0	8.3	0.93
	5.0+3.1	8.1	0.62
D	5.4+2.7	8.1	0.50
	5.0+3.1	8.1	0.62
E	3.8+3.1	6.9	0.82
	3.5+2.7	6.2	0.77
F	4.2+1.9	5.1	0.45
	5.0+2.3	7.3	0.46
G	3.5+1.5	5.0	0.43
	3.4+1.5	4.9	0.44
H	4.6+0.38....(t)	5.0	0.08
	5.0+0.38....(t)	5.4	0.08

(t): satellite

CHAPTER V. SEROLOGY

Systematic serology of plants is an method whereby the production of antibodies in a test animal is used to establish presence or absence of compounds in plants. Proteins extracted from seeds or pollen are most frequently used. When foreign proteins (i.e., antigens) are injected into an animal, its immune system responds by producing antibodies specific to those antigens. These antibodies are then reacted with antigens from other taxa. The degree of this reaction is an indication of antigen similarity between the taxa.

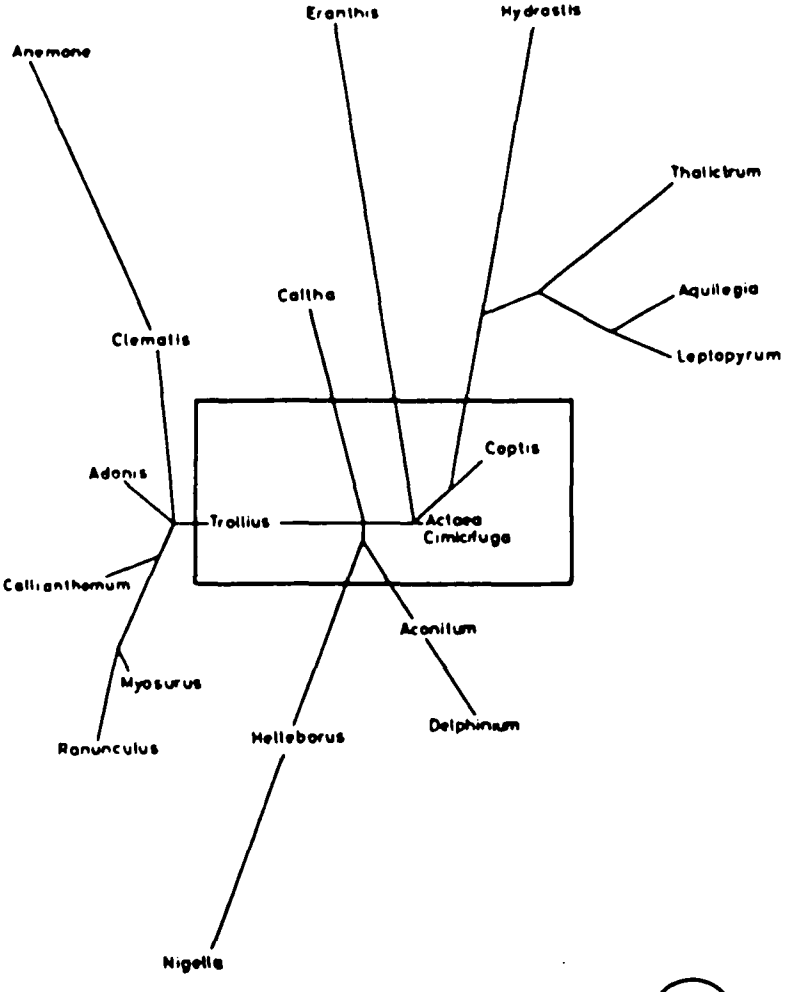
Since morphological characters are end-products of complex interconnected biochemical pathways which are under the control of a number of genes and external signals, it is pertinent to analyze proteins which are known to be primary gene products (Sengbusch, 1983) and to study these proteins in order to solve problems in phylogeny. Similarities and dissimilarities are believed to reflect evolutionary changes (Sengbusch, 1983). Using immunological techniques as analytical tools, it has been shown that proteins are excellent markers for tracing protein evolution. Hammond (1955) conducted a serological investigation to examine primarily the problem of subfamilial relationships within the Ranunculaceae. The precipitin reaction was measured using turbidity, that is, by the generation of Boyden curves. The areas under the curves are then compared to those in the reference reactions in order to compute

serological correspondence. Hammond (1955) was concerned with comparing serological results with relationships inferred from morphological information (using primarily the character states of achenes versus follicles) and chromosome size. Results were given for antisera from several species reacting with antigens of about 20 genera. He found that serological correspondence did not support the earlier taxonomy. Over a decade later, Jensen (1968) continued and expanded the tradition of serotaxonomic research with a survey of the relationships and classification of the genera in the Ranunculaceae. In a survey of 20 ranunculaceous genera, he compared the proteins of mature seeds by serological methods, producing a serological phylogeny of the genera. The results were presented in a two-dimensional scheme of generic affinities (Figure 3). Based on these data, it was suggested that Cimicifuga, Actaea, Coptis, and Trollius, which are in the box of Figure 3, are closely related and are the most ancestral genera. Jensen did not study Anemonopsis, Beesia and Souliea, but other studies (Tamura, 1968; Pellmyr, 1985) suggest a position of these genera between Actaea/Cimicifuga and Trollius. Jensen and collaborators (Jensen & Butter, 1981; Jensen & Greven, 1984) have carried out serological investigations utilizing partially purified storage proteins. These workers suggested that the seed storage proteins of dicots consist largely of two proteins. These observations are important for serological studies because they suggest that homologous proteins may be compared across the dicots. Later, Jensen and Greven (1984)

purified the legumin-like protein from Magnolia tripetala and used it to produce antisera. Serological similarities among members of the Ranunculaceae were examined again by using whole seed protein extracts and the antisera from Magnolia. Since Magnolia is assumed to be one of the most primitive angiosperms, this protein can be considered to be phylogenetically primitive and the results could indicate the degree of protein advancement and thus might indicate primitive or advanced taxa within the Ranunculaceae. The results are in agreement with the evolutionary classification for the genera of the Ranunculaceae proposed by the data using the complete soluble seed protein system (Jensen, 1968) and proposed by modern karyological and chemical data (Gregory, 1941; Ruijgrok, 1967). Also, the similarities within (sub)tribes are demonstrated serologically e.g., of Coptis and Xanthorhiza (Coptidinae), of Cimicifuga and Actaea (Actaee), of Thalictrum and Aquilegia (Thalictreae). Secondly, the tribes Coptideae and Actaeeae, and the genus Trollius, reveal the highest similarity to the Magnolia reference protein, indicating that these genera might have maintained most of the Magnolian, i.e. primitive, protein characters. Thus it can be inferred that the Coptideae and Actaeeae are phylogenetically the most primitive tribes within the Ranunculaceae because they occupy a central position in the serological similarity system of the family proposed by Jensen (1968) (Figure. 3). The designated derived genera of the Ranunculaceae, Delphinium, Aconitum, Helleborus, Consolida, Ranunculus, Clematis, and Nigella,

show less serological correspondence to Magnolia. Eranthis did not react at all, which indicates a distant position (Jensen, 1971). Thirdly, these data again confirm the serological similarity between the Coptideae and Actaeeae (see Jensen, 1968) even though the karyological characters (9 small chromosomes with distinct heterochromatin in Coptideae, generally 8 large chromosomes and irregularly distributed heterochromatin in Actaeeae [Langlet, 1932; Gregory, 1941; Stebbins, 1971]) and chemical characters (e.g. benzylisochinoline-alkaloids; Ruijgrok, 1967) definitely separate them. However, up to now, no one has been able to include intermediate genera between tribe Trollieae and tribe Cimicifugeae in studies aimed at understanding the phylogeny. The intermediate genera are small Asian genera that represent critical taxa between Trollius and the other central genera. The close examination of these Asian genera is necessary. Jensen and Grumpe (1983) suggested that seed storage proteins have proved to be valuable macromolecules for the elucidation of phylogenetic relationships and this has been especially true for serological investigation. The serological studies of these Asian genera is not completed.

Figure 3. Serological phylogeny within Ranunculaceae from Jensen (1968). Box shows closely related, most ancestral genera. Tamura (1968) and Pellmyr (1985) suggested that other studies suggest the Asian genera would positioned between Actaea/Cimicifuga and Trollius.


Eranthis
Hydrastis
Anemone
Thalictrum
Caltha
Aquilegia
Clematis
Leptopyrum
Coptis
Adonis
Trollius
Actaea
Cimicifuga
Callianthemum
Aconitum
Myosurus
Delphinium
Ranunculus
Helleborus
Nigella

3

CHAPTER VI. CHLOROPLAST DNA ANALYSIS

Introduction

One of the goals of systematics is to reconstruct evolutionary trees of organisms as accurately as possible. One approach to this problem is to examine the fossil record, and morphological and anatomical characters. Using this approach, classical evolutionists were able to infer the major aspects of evolution. However, the evolutionary change of morphological and anatomical characters is usually so complex that this approach does not produce a clear-cut picture of evolutionary history, and the details of the evolutionary trees were almost always controversial.

The situation changed in the mid-1960s when molecular techniques were introduced into the study of evolution. In recent years, there has been increased use of molecular techniques in the study of evolution. The techniques most commonly used are gene cloning, rapid DNA sequencing, and restriction-enzyme analysis. Owing to these developments, the 1980s has seen an explosion in the use of molecular data for the study of evolutionary problems. The field of plant molecular evolution has participated in this expansion, especially where the chloroplast genome (cpDNA) is concerned (Clegg & Zurawski, 1992).

Chloroplasts are intracellular organelles present in higher plants and algae, which contain the entire enzymic machinery for the process of photosynthesis (Shinozaki, et al., 1986). The chloroplast genomes of land plants are circular DNA molecules ranging from 120 to 190 Kilobase pairs (Kbp). The chloroplast genome contains, with few exceptions, two duplicate regions in reverse orientation, known as the inverted repeat (IR). These repeated regions separate the remainder of the molecule into large single-copy (LSC) and small single-copy (SSC) regions. (Figure.4)

The advantages of the chloroplast genome for evolutionary research are: 1) a relatively accessible component of plant total DNA, and even with a small amount of material, it is possible to obtain enough chloroplast DNA to get abundant information; 2) an extensive background of molecular information on the chloroplast genomeexists, and there are now enough data available on the cpDNA of land plants to make it possible to study distantly related groups; 3) the conservative rate of nucleotide substitution in the cpDNA sequence change potentially allows resolution of phylogenetic relationships at higher taxonomic levels; 4) DNA sequence data is independent of other biological characters, therefore no assumptions about relationships are necessary to infer phylogenies from sequence data, and 5) the feature of uniparental inheritance is valuable and allows you to study the origin of species via hybridization.

With the above in mind, I studied the cpDNA of 15 species. This is the first time such studies have been done on Cimicifugeae. The construction of restriction site maps, based on single and double restriction digests, is an essential prerequisite of the work (Johansson & Jansen, 1991), and restriction site maps from a large number of plant species have been published. I first examined the overall structure of cpDNA of Cimicifuga simplex, a representative of the tribe Cimicifugeae, to determine the feasibility of using restriction site data in phylogenetic analyses.

Materials and Methods

Estimation of the molecular sizes of restriction fragments.

Plant material was obtained as fresh leaf material and brought back to the laboratory and preserved at 4°C for 4 - 5 days in the dark to reduce the amount of starch in the leaves. DNA was isolated from fresh leaf material as purified chloroplast DNA by the sucrose gradient method (Palmer, 1986). (Appendix 3) All cpDNAs were further purified by CsCl/ethidium bromide gradient centrifugation. The six-base-pair sequence restriction endonucleases Pst I, Mlu I, and Xho I, which digest cpDNA rarely but digest nuclear DNA extensively, were used for estimation of the molecular sizes of restriction fragments. Mapping digests consisted of single digests (with Pst I, Mlu I, and Xho I) and double digests (with Mlu I+Xho I and Pst I+Mlu I). The digested fragments were separated on the basis of size by electrophoresis in 0.7 % standard agarose (Nippon gene) and in 0.3 % high melting agarose (Nippon gene). Size markers used were lambda DNA digested with Sty I for 0.7% standard agarose and equimolar mixtures of T4dC(+) and T4dC digested with Bgl I for 0.3% high melting agarose respectively. After the fragments were separated on a gel, the restriction fragment patterns were viewed with transmitted UV light and photographed. The mobility of each

fragment from the origin was measured, based on which its molecular size was estimated.

Physical mapping of Cimicifuga simplex cpDNA.

A restriction site map of Cimicifuga simplex cpDNA based on single and double restriction digests has been constructed employing the molecular hybridization method of Southern (1975) and non-radioactive, the ECL direct nucleic acid labelling and detection system based on enhanced chemiluminescence (ECL) (Amersham). The procedures of ECL system is shown in Appendix 4. The system allows you to label a probe DNA with horseradish peroxidase and a set of tobacco probes (Figure 5) was obtained from Gene Research Group of Nagoya University in Japan. This is achieved by peroxidase with a positively charged polymer added and is attached to a single-stranded form of probe DNA by charge attraction. Addition of glutaraldehyde causes the formation of chemical cross-links so that the probe is covalently labelled with enzyme. Once labelled, the probe is used in hyridization with target DNA immobilized on a membrane. After hybridization, the membrane is washed, then taken into the ECL. detection step. There are two detection reagents that react with enzyme to produce blue light; first detection reagent decays to hydrogen peroxide and reduction of hydrogen peroxide by the enzyme is coupled to the light producing reaction by second

detection reagent. Therefore, the bands can be detected on a blue-light sensitive film.

Results

From the mapping digests, the cpDNA size of Cimicifuga simplex is estimated to be approximately 150 Kb. The results of electrophoresis are shown in Figures 6 and 7, and the identified restriction fragments and their sizes are given in Table 7. A map of the overall structure of cpDNA was constructed based on the restriction site data but is not shown here due to an insufficient collection of proves to cover the entire cpDNA genome.

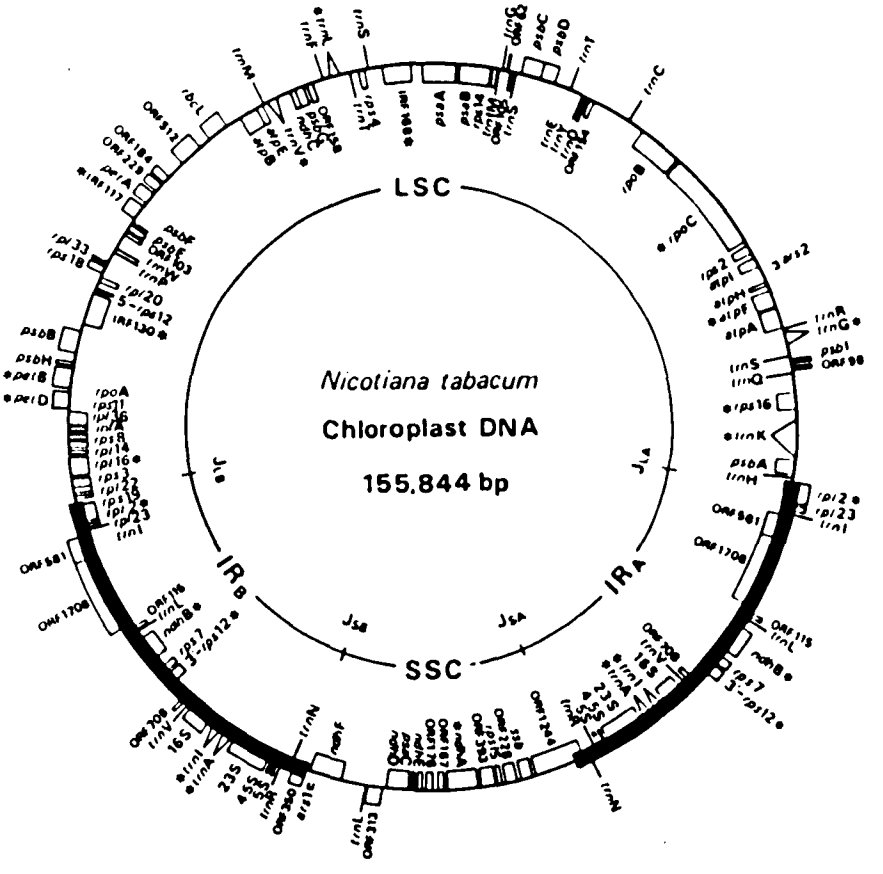
There is some size variation found among cpDNAs from different genera in the Ranunculaceae. In Caltha, Aquilegia, Delphinium, and Ranunculus the cpDNA sizes have been estimated at 154, 151, 149, and 157 Kb, respectively (Johansson & Jansen, 1991; Palmer, 1990). Cimicifuga has approximately 150 Kb which is the average size in the family. The occurrence of structural rearrangements in the cpDNA also varies within the family (Johansson & Jansen, 1991). Inversions have been found in Anemone, Adonis and Clematis (Hoot & Palmer 1990, Palmer, 1990), whereas most Ranunculaceae lack inversions (Johansson & Jansen, unpubl.).

Johansson & Jansen (1991) concluded that cpDNA restriction site data provide valuable information on the phylogeny of the Ranunculaceae, both at interspecific and intergeneric levels.

However, they also said that intergeneric restriction site comparisons are feasible only among genera whose genomes are not rearranged. Certain regions of the chloroplast genome cannot be compared due to extensive length variation, which makes it impossible to reliably align restriction sites.

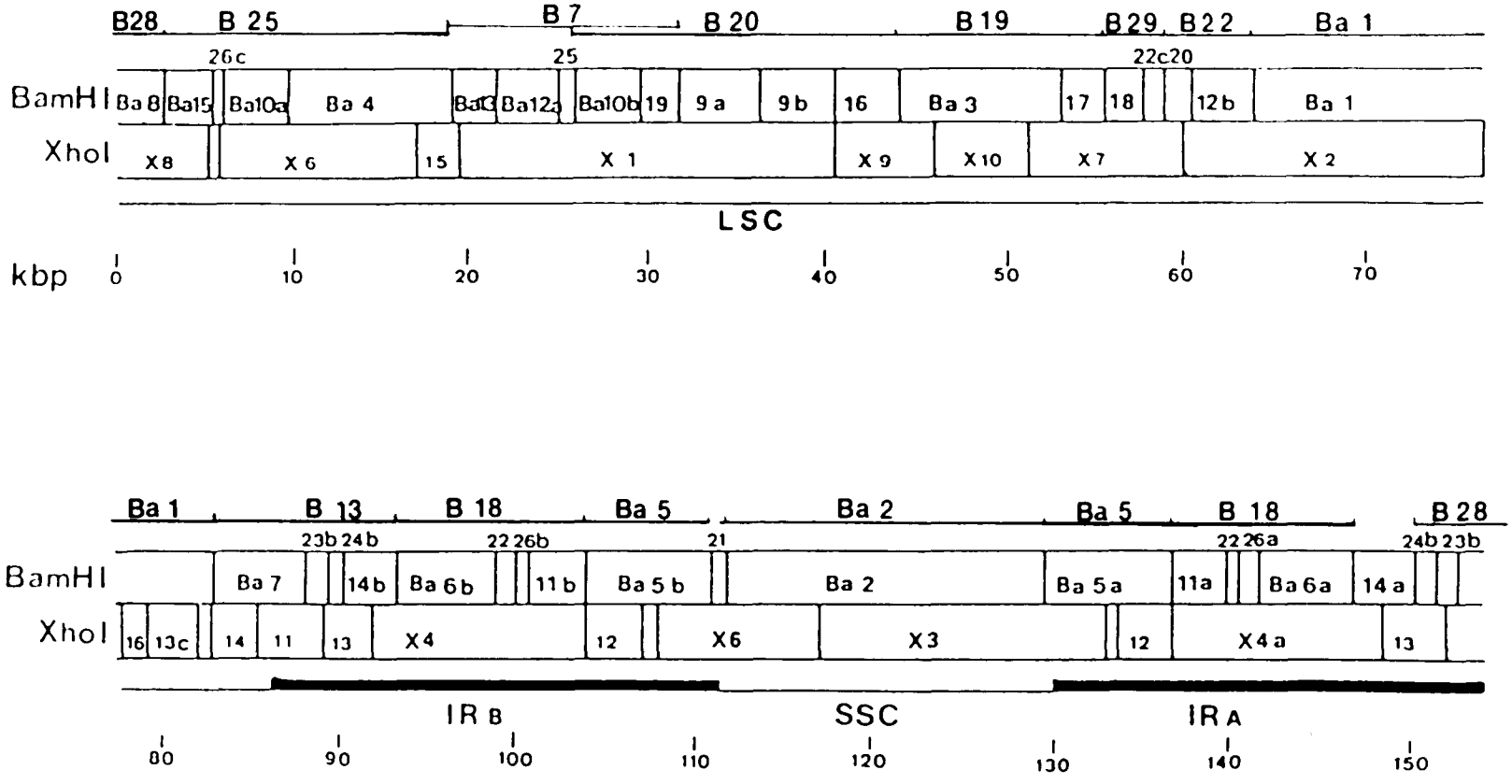
Although Cimicifuga did not show structural rearrangements in cpDNA, when I conducted analyses of cpDNA restriction sites using 10 restriction enzymes with the seven genera (in the tribe Cimicifugeae and closely related genera), I could not interpret the bands clearly probably due to the reason above. In addition to the difficulty in interpreting the restriction sites, I did not have enough fresh leaves to extract large amount of DNAs of the Chinese genera to carry out further work with restriction enzymes. I then moved to work with sequencing the *rbc*L gene in cpDNA using the PCR method to amplify the materials from my limited samples.

Figure 4. Circular gene map of the tobacco chloroplast genome. One of the outstanding features of chloroplast DNAs of most higher plants is the presence of large inverted repeats (IR) shown by bold lines. The IR sequences are separated by a large and a small single-copy region (LSC and SSC, respectively). The figure is taken from Shinozaki et al. (1986).


LSC
IRB
IRA
SSC
Nicotiana tabacum
Chloroplast DNA
155,844 bp

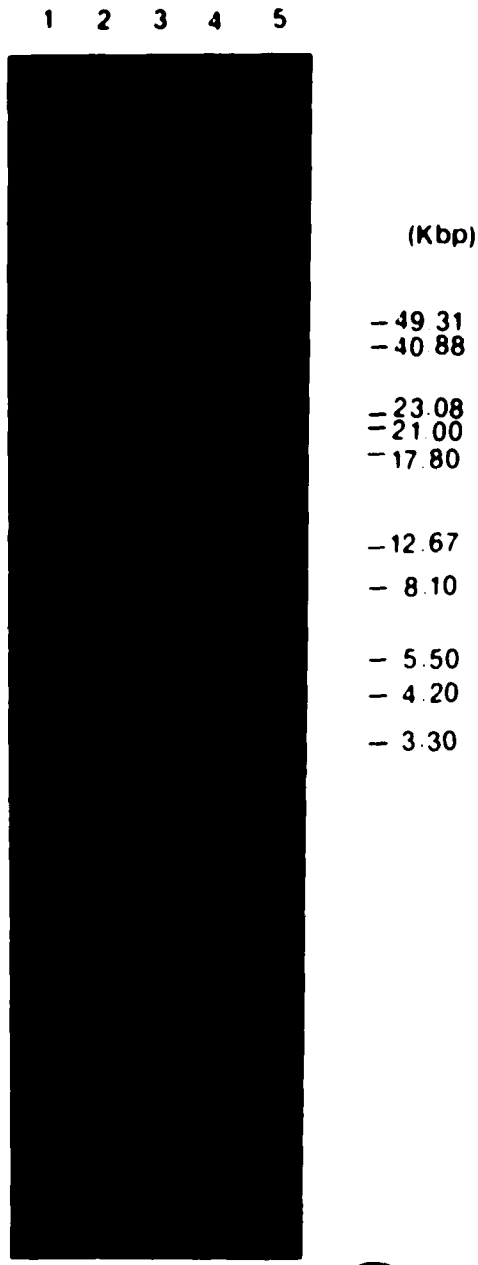
4

Figure 5. Physical map and cloned fragments of tobacco chloroplast DNA. The Bam HI (Ba) and Xho I (X) fragment maps are presented in a linearlized form. The cloned fragments used as probes are indicated by lines with plasmid names (the prefix pT is omitted) in the upper part. B is Bgl I and Ba is Bam HI respectively.


B28
B 25
B 7
B 20
B 19
B 29
B 22
Ba 1
26c
25
22c
20
BamHI
Ba 8
Ba15
Ba10a
Ba 4
Ba13
Ba12a
Ba10b
19
9 a
9 b
16
Ba 3
17
18
12b
Ba 1
XhoI
X 8
X 6
15
X 1
X 9
X10
X 7
X 2
LSC
kbp
0
10
20
30
40
50
60
70
Ba 1
B 13
B 18
Ba 5
Ba 2
Ba 5
B 18
B 28
23b
24b
22
26b
21
22
26a
24b
23b
BamHI
Ba 7
14b
Ba 6b
11 b
Ba 5 b
Ba 2
Ba 5a
11a
Ba 6a
14 a
XhoI
16
13c
14
11
13
X4
12
X6
X3
12
X4 a
13
IR B
SSC
IR A
80
90
100
110
120
130
140
150

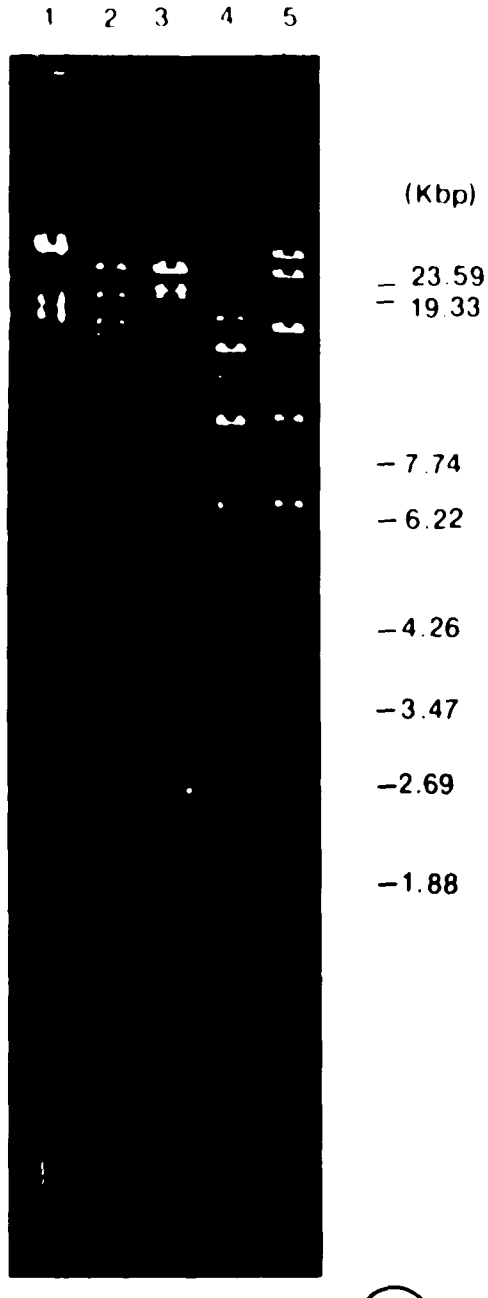
5

Figure 6. Separation of Cimicifuga simplex cpDNA Pst I, Mlu I and Xho I on a 0.3% high melting agarose gel. Size in kb is indicated for selected lambda DNA reference fragments shown in the lane to the right. Lane 1 is a single digest by Pst I, lane 2 is double digests by Pst I and Mlu I, lane 3 is a single digest by Mlu I, lane 4 is double digests by Mlu I and Xho I, and lane 5 is a single digest by Xho I respectively.



6

Figure 7. Separation of Cimicifuga simplex cpDNA Pst I, Mlu I and Xho I on a 0.7% low melting agarose gel. Size in kb is indicated for selected lambda DNA reference fragments shown in the lane to the right. Lane 1 is a single digest by Pst I, lane 2 is double digests by Pst I and Mlu I, lane 3 is a single digest by Mlu I, lane 4 is double digests by Mlu I and Xho I, and lane 5 is a single digest by Xho I respectively.



(7)

Table 8. Molecular size and copy number of the restriction fragment of Cimicifuga simplex cpDNA generated by single or double digestion with Pst I, Mlu I, and Xho I.

	Pst I	Pst I+Mlu I	Mlu I	Mlu I+Xho I	Xho I
1	45.89	25.02	25.02(x2)	17.67	40.43
2	42.50	20.06	21.50	15.17(x2)	24.52
3	19.32	13.50	20.06	12.20	14.05(x2)
4	16.75	12.80	11.60	9.50(x3)	10.20(x2)
5	13.50	11.60	11.20	6.50(x3)	6.60(x2)
6	12.80	10.60	10.60	5.80(x2)	5.80
7		8.00	9.50	5.50	5.20
8		7.50	5.80	5.30	3.85
9		5.80	4.20 (x2)	5.20	3.47(x2)
10		5.40	1.80	3.47(x2)	1.20
11		4.20(x3)	0.83	2.20	
12		3.80		1.65	
13		1.88		1.20	
14		1.00		0.66	
15		0.74			
Total	150.76	150.30	151.33	148.46	149.64

Mean of cpDNA: 150.10 Kbp

Table 9. Southern hybridization of Tobacco probes to single or double digest of Cimicifuga simplex cpDNA with Pst I, Mlu I, and Xho I.

Tobacco probe	Hybridization to				
	Pst I	Pst I+Mlu I	Mlu I	Mlu I+Xho I	Xho I
1	1	1,6,12	1,2,6	1,4,(5),10	2,(5),8
2	1	2,12	3,9	4,8,10	4,9
5	1	2,5	3,4	4,9,10	4,7,8
7	3,5,6	8,9,11,12	5,7,8	4,6,10	1
13	4,5,6	3,4,12	1,2,7	2,5	3,5
18	2,5,6	3,4,(5),11	1,2,4	2,10	3
19	2	1	1	1,5,6	2,5,6
20	1,4	1,8,9,10,11	1,5,8,9	3,5,6,10	1,5
22	1	1	1	1	2
25	3	7,8,12	1,7,9	4,7,10	1
28	5,6	3,4,7	1,2	2,5	2,5
29	1	1	1	1	2

CHAPTER VII. *rbc* L SEQUENCING

Introduction

One of the most important achievements in the study of molecular evolution is the discovery of the approximate constancy of the rate of amino acid or nucleotide substitution (Nei, 1987). This discovery has provided evolutionary biologists with a new tool for constructing phylogenetic trees. The constancy of the rate of amino acid or nucleotide substitution holds only approximately, but compared with morphological or physiological characters, molecular data show a much more regular pattern of evolutionary change. In addition, in the past two or three decades, many statistical methods have been developed for analyzing and constructing quantitative trees. Owing to the development of these methods, it is expected that they could give a clearer picture of the evolutionary relationship of organisms than do morphological characters. A primary objective of phylogenetic studies is to reconstruct the evolutionary history of a group of organisms. In spite of the time, effort, and expense involved, DNA has been sequenced more and more frequently in systematic and phylogenetic studies. Presently, there are two methods that are widely used to determine DNA sequences, the enzymatic dideoxy method and the chemical method, differing primarily in the technique used to generate the ladder of oligodeoxynucleotides. The dideoxy method is preferred by many

people. This method utilizes a DNA polymerase to synthesize a radiolabeled, complementary copy of a template in the 5'->3' direction and, after a dideoxy nucleotide is incorporated, terminates chain elongation selectively at A, T, G, or C, thereby generating the ladder of oligodeoxynucleotides resolved on gel.

Perhaps the most difficult problem is the choice of an appropriate gene and the choice of which algorithm to use in analysis of the data. There are several versions of parsimony methods, but the principle of this method is primarily for constructing a topology, and it is intended to construct a 'realized' tree rather than an 'expected' tree (Nei, 1987). Nucleotide sequence data, which can give a more refined picture of deduced relationships, should prove to be effective. In this chapter I present the results of phylogenetic analyses using the sequences data of the chloroplast gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc* L). This gene codes part of a key enzyme which fixes CO_2 in the Calvin-Benson cycle of photosynthesis and also plays a role in photorespiration in C_3 plants. The complete nucleotide sequence (155,844 bp) of tobacco chloroplast DNA has been determined (Shinozaki et al., 1986), of which the tobacco rbcL gene coding region contains 1431 bp (477 codons).

One might assume that any polymorphic site in which two or more different types of nucleotides exist is useful for determining the topology in the construction of maximum parsimony trees, however, this is not the case, and any nucleotide that exists

uniquely (i.e., as an autapomorph) in one operational taxonomic unit (OTU) is not informative. This is because such a nucleotide can always be assumed to have arisen by a single mutation in the immediate branch leading to the OTU in which the nucleotide exists, and the mutational change is compatible with any topology. A nucleotide site is informative only when there are at least two different kinds of nucleotides, each represented at least two times. For example, the second nucleotide site in Appendix 1 is noninformative, whereas the nineteenth nucleotide site with a dot is informative.

Zurawski et al. (1984) examined the nucleotide sequences of three genes from barley and maize and obtained a mutation rate of 1.1×10^{-9} per site per year. Because of this low rate of evolution, this gene appears to be useful at higher taxonomic levels (among distantly related genera within a family and higher) and currently it is being used to examine evolutionary relationships in a wide diversity of plant groups. Fourteen species of Ranunculaceae, one species whose position in the Ranunculaceae has been questioned, and two outgroup families, Berberidacae and Menispermaceae, were included in the analysis.

Materials and Methods

Plant material was field-collected. Species names and the collected localities are listed in Table 10. Voucher specimens are

preserved in the herberium of Kanazawa University (KANA) and also all the plants are potted and grown in the greenhouse at the Kanazawa University. I sequenced 15 species of Ranunculaceae and sequence data for the outgroups were obtained from the Kanazawa University Gene Research Insititute where I conducted the laboratory work.

A cloning strategy was employed for generating sequence data. Total DNA was isolated from each species using the CTAB (hexadecyltrimethylammonium bromide) method (Doyle & Doyle, 1987a; Doyle & Dickson, 1987b) shown in Appendix 5. A segment of the chloroplast genome containing the rbcL gene was amplified by the polymerase chain reaction (PCR) using Taq polymerase and two synthetic primers (Figure 8a). The 5' primer (1 - 1) is based on the first 26 nucleotide positions of the rbcL coding sequence, previously published, of Nicotiana tabacum and Marchantia polymorpha (Shinozaki et al., 1986; Ohyama et al., 1986). The 3' primer (3 - 2) is based on a 27 bp sequence that contains the sequence position of 1360 - 1386 for Nicotiana and Marchantia. (Figure 8a). This procedure (Appendix 6) yielded double-stranded segments of approximately 1386 basepairs (bp). Samples that failed to amplify segment, due to the many secondary compounds being produced in a leaf, were run through a Nick Column (Pharmacia) to purify the DNA before doing PCR. The amplified fragments containing *rbc* L were gel-isolated from 1.0% low melting agarose (Nippon Gene). The fragments were kinased and then ligated directly to Sma I digested

pUC 19 vectors. The constructs were used to transform E. coli strain JM 109 (transformation kit from Nippon Gene) and recombinant white colonies were selected on ampicillin/X-gal plates. Five to ten white colonies were taken from each plate for the quick screen method (phenol-chloroform extraction) and the desired clones were grown in a LB medium. The bacteria multiply in the medium, as do the plasmids, and the latter produce large amounts of the DNA of interest. All plasmid DNAs were isolated from bacteria by alkaline-SDS extraction procedure of Birnboim (1983), (Appendix 7). The entire cloned fragment was sequenced by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase Version 2.0 (US Biochemicals) and a series of eight overlapping synthetic primers and universal primers for pUC 19 (Figure 8b). DNA sequencing techniques are based on polyacrylamide gel electrophoresis. In this work, I used a gel (Hydro-Link Long Ranger, AT Biochem) which contains a chemically modified acrylamide monomer with a novel cross-linker. This Long Ranger gel allows you to obtain more sequencing information when compared to equal percentage polyacrylamide gels run for an equivalent time. Eight of the synthetic primers were constructed at the Kanazawa University Gene Research Insititute using sequences from the several species of Lardizabalaceae, one species of Berberidaceae, and one species of Menispermaceae. pUC 19 clones were sequenced from the double-stranded template, and then the consensus sequence for each clone was taken. Alignment of sequences was done by

comparison to the sequence for Nicotiana tabacum with GENETYX Ver.6.0 (1987, 1988). I repeated this whole procedure to avoid mistakes from the PCR by taking the consensus sequence between the first clone and the second clone. When the first clone and the second clone showed different sequences, a third clone was used to verify the sequences.

Phylogenetic analyses were conducted and compared by using two parsimony methods: PAUP Version 3.0c (Swofford, 1989) and Hennig 86 (Farris, 1988). All 1333 bp of nucleotide sequences were used to make the data matrix for PAUP 3.0c. The data matrix for Hennig 86 was generated based on the data matrix produced by PAUP 3.0c and the letters for DNA were transformed to numbers in which, 0=A, 1=T, 2=C, and 3=G, respectively. Character state changes were unordered and unweighted in this analyses. Parsimony analyses using PAUP 3.0c were conducted using the Branch and Bound option to find all most parsimonious trees. The bootstrap method (Felsenstein, 1985) was employed to evaluate the reliability of phylogenetic estimates and five hundred replicates were performed. MacClade (version 2.97c+, Maddison & Maddison, 1989) was used in conjunction with PAUP to explore character state evolution and alternative topologies. In addition, I expericmented with comparisons between nucleotide data vs. amino acid data, and to check the confidency of the two outgroups used by using either one of them as a outgroup and then using both as an outgroup.

Results

A total of 1333 bp of sequence (beginning at position 27 and ending at position 1359) was obtained (Appendix 1). Of the 1333 bp compared, a total of 247 variable nucleotide positions was identified and 34, 27, and 186 of these occurred at the first, second, and third codon positions, respectively.. Of the variable positions, 120 were phylogenetically informative (Table 11). The ratio of informative mutations by codon position was 17: 6: 97 respectively. The ratio of DNA contents in the *rbc* L gene are shown in Table 12.

Analyses using PAUP

Parsimony analyses of the phylogenetically informative base substitutions using the data matrix of Table 11 with the Branch and Bound search resulted in three equally parsimonious trees of 360 steps and a consistency index of 0.641. The strict consensus tree is shown in Figure 9. The number below the internal branches indicate the percentage of times that the branch was recovered in 500 bootstrap samples.

Analyses using Hennig 86.

The analysis was run by using the data matrix shown in Table 13 and only one outgroup (Caulophyllum) was used. Hennig 86 is similar to PAUP in using the parsimony algorithm but it differs by not accepting polymorphism. This method was employed to examine

the accuracy of the topology generated with PAUP shown in Figure 10. The result showed the same topology, and the reliability of the topology generated with PAUP was confirmed.

Amino Acid data.

Since my 1333 bp nucleotide sequence started at the 27th position, I used the second nucleotide position of the 1333 bp to translated to an amino acid sequence. After the translation, I obtained 444 amino acids (Appendix 2) of which only 11 informative amino acid characters were scored (Table 14). Because this is not enough characters for analysing the 16 taxa so the analyses using amino acid were not made. Thus, a comparison of nucleotide data vs. amino acid data was not possible.

About the outgroup.

I used two outgroup suggested by Cronquist (1981) and Loconte & Stevenson (1991), interchangably or used both together to test if the outgroup choice was influencing the topology. The three different trees are generated by different outgroups and the result (Figure 11a, b, and c) showed that the interchanging of outgroups did not change the original topology generated from using both outgroups. This result indicates that the Ranunculaceae are a monophyletic family which were supported by several anatomical and chemical characters.

The rapidly increasing use of molecular data has focused attention on the pros and cons of molecular versus morphological evidence. In the next chapter, a general comparison of molecular and morphological studies is explored.

Table 10. Material collected for rbcL sequence analysis.

Taxon	Source *	Chromo. no. (2n)	tribal group **
Ranunculaceae			
Actaea asiatica	Nagano, Japan	16	Cimicifugeae
Adonis amurensis	Nikko Bot. Garden[1)]	32	Ranunculeae
Anemonopsis macrophylla	Nikko Bot. Garden[1)]	16	Cimicifugeae
Beesia calthifolia	Omei-san, China	16	Trollieae
Caltha palustris var. enkoso	Nikko Bot. Garden[1)]	16	Trollieae
Cimicifuga simplex	Nikko Bot. Garden[1)]	16	Cimicifugeae
Clematis terniflora	Saitama, Japan	16	Anemoneae
Coptis japonica	Nikko Bot. Garden[1)]	18	Coptideae
Eranthis pinnatifida	Chichibu, Japan	16	Helleboreae
Isopyrum nipponicum	Kanazawa, Japan	14	Isopyreae
Souliea vaginata	Markang, China	16	Cimicifugeae
Thalictrum minus var. hypoleucum	Kanazawa Univ. [2)]	14	Thalictreae
Trollius riederianus	Norikura, Japan	16	Trollieae
Xanthorhiza simplissisima	Arnold Arboretum [3)]	36	Coptideae
Hydrastis canadensis	Kanazawa Univ. [2)]	26	Hydrastideae
Berberidaceae(outgroup)			
Caulophyllum robustrum	Hakusan, Japan	18	——
Menispermaceae(outgroup)			
Cocculus trilobus	Togakushi, Japan	50	——

*

1) Nikko Branch Garden, Botanical Garden, Faculty of Science, University of Tokyo, Nikko, Tochigi Perfecture, Japan.
2) Cultivated plants in Green house of the Botanical Garden in Kanazawa University, Kanazawa, Japan.
3) Arnold Arboretum, Harvard University, Boston, U.S.A.

**

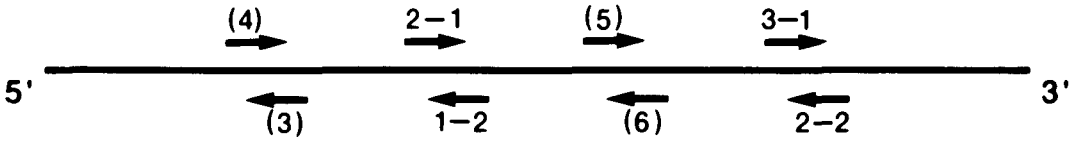
Classification of Tamura (1966, 1968)

Figure 8. Synthetic oligonucleotides used as amplification primers and overlapping primers used in dideoxy sequencing. The number in parentheses indicates the position of primer within the gene.

(a) Two synthetic primers used to amplify the nucleotide fragment which continue *rbc*L gene.
(b) Two universal primers for pUC 19 and eight overlapping synthetic primers used in dideoxy sequencing.

1-1 (1 - 26) 5' ATGTCACCACAAACAGACACTAAAGC 3'
3-2 (1360 - 1386) 5' CCATACTTCACAAGCAGCAGCTAGTTC 3'

(a)



primer for vector
primer-40 5' GTTTTCCCAGTCACGAC 3'
primer 3 5' GGAAACAGCTATGACCATG 3'

1-2 (544-571) 5' CATAAACGGCTCTACCGTAGTTTTAGC 3'
(3) (358-380) 5' ATTGTGGGTAATGTATTTGGGTT 3'
(4) (283-302) 5' AATCAATATATTTGTTATGT 3'
2-1 (496-521) 5' GGTCGTCCCCTGTTGGGATGTACTAT 3'
2-2 (1099-1123) 5' GAACACCTGGTAAAGAGACCCAATC 3'
(5) (817-836) 5' GGAATCACTGCAAATACTAG 3'
(6) (894-913) 5' TGCAGTTATTGATAGACAGA 3'
3-1 (1030-1054) 5' GGCTTTGTTGATTTACTGCGTGATG 3'

(b)

Table 11. Seventeen taxa scored for 120 potentially informative rbcL characters (invariant sites and autapomorphies removed), with characters numbered by base position within the gene. All positions are third positions within codons, unless otherwise indicated above the character number. The complete set of potentially informative nucleotides is given for Actaea; a nucleotide is shown for other taxa only if it differs from Actaea (a dot indicates that the same nucleotide is present). Missing information is indicated by "?".

			(2)			(1)	(2)	(1)			(1)																			
	1	1	2	4	5	5	6	6	6	6	8	8	8	8	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3	3
	0	9	2	2	5	8	2	3	5	7	1	3	5	8	2	1	2	3	9	2	3	5	1	6	2	9	1	4	5	6
Actaea	C	A	T	C	T	A	G	C	C	C	C	T	G	G	T	A	T	A	T	C	C	T	T	A	C	C	C	A	C	?
Adonis	.	.	.	.	G	.	.	A	.	A	.	.	.	.	G	C	.	.	.	.	.	C	.	.	.	.	.	.	.	.
Anemonopsis	.	.	.	.	T	.	.	C	.	C	.	.	.	.	T	A	.	.	.	.	.	T	.	.	.	.	.	.	.	G
Beesia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C
Caltha	.	.	.	A	.	.	A	.	.	.	.	.	.	A	.	.	.	A	.	.	.	.	C	.	.	.	.	.	.	G
Cimicifuga	.	.	.	C	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	G	.	.	.	T	.	.	?
Clematis	.	.	.	A	.	.	G	A	A	.	.	.	.	.	.	.	.	.	.	.	T	C	C	.	.	.	.	.	.	G
Coptis	.	G	C	.	.	.	A	C	C	.	.	C	A	A	.	G	G	G	.	.	C	.	.	.	.	.	C	T	.	?
Eranthis	.	A	T	.	.	.	G	.	.	.	.	T	G	G	.	A	T	A	.	T	T	T	T	.	.	.	.	A	.	G
Isopyrum	.	G	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	G	.	C	A	C	C	.	.	.	.	.	.	.
Souliea	.	A	.	C	.	.	A	.	.	.	.	.	.	.	T	.	.	A	.	.	C	T	T	.	.	.	.	.	.	.
Thalictrum	.	G	.	A	.	.	G	T	A	.	.	.	.	.	G	.	.	.	.	.	.	C	C	.	.	.	.	.	.	?
Trollius	.	A	.	.	G	.	.	A	C	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
Xanthorhiza	.	G	C	C	T	.	A	C	.	C	.	C	A	A	T	.	G	G	.	.	.	.	.	.	.	.	.	T	.	C
Hydrastis	.	.	T	A	.	.	G	.	.	.	.	T	G	.	.	.	T	A	C	.	.	.	.	.	.	.	.	A	.	?
Caulophyllum	A	.	.	C	.	C	.	T	A	.	T	.	.	.	G	G	.	.	.	T	.	T	T	C	T	T	T	.	T	C
Cocculus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G

Table 11. continued.

	(1)				(1)		(1)																(1)	(1)	(2)						
	3	3	3	3	3	3	3	4	4	4	4	4	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	7	7
	6	6	6	7	7	9	9	2	2	3	3	7	1	2	3	3	5	6	2	1	0	4	4	5	5	6	6	7	9	2	4
	2	4	8	6	7	4	8	1	4	3	6	2	7	9	2	5	6	2	1	4	0	7	0	1	1	4	0	1	7	7	5
Actaea	?	A	G	T	T	T	G	A	A	T	C	T	C	C	C	A	C	G	A	C	T	A	T	A	A	C	A	C	G	C
Adonis	.	?	.	.	.	.	.	.	T	C	T	C	T	.	T	.	.	A	.	.	.	.	.	.	.	.	G	.	A	T
Anemonopsis	C	.	?	.	C	C	.	.	A	T	C	T	.	T	C	.	.	G	.	.	.	.	.	.	.	.	A	.	.	C
Beesia	?	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Caltha	C	A	G	.	.	T	.	.	C	.	.	.	C	C	T	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.
Cimicifuga	?	?	?	.	.	C	.	.	A	.	.	.	T	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
Clematis	T	A	G	.	.	T	.	.	C	.	.	.	.	T	T	.	T	A	.	.	.	A	.	.	.	.	.	.	.	.
Coptis	C	?	?	.	T	.	.	G	A	.	T	.	.	C	.	.	C	.	.	A	.	.	.	.	.	.	C	.	G	.
Eranthis	A	A	G	.	C	C	.	A	.	.	C	.	.	.	C	.	.	G	.	C	.	.	.	T	.	.	A	.	A	.
Isopyrum	C	G	?	C	.	T	.	.	C	.	G	.	C	.	T	.	.	A	G	.	.	.	.	A	.	.	.	T	.	T
Souliea	.	?	.	T	.	A	.	.	A	.	C	.	T	.	C	.	.	G	A	.	.	.	.	.	.	.	.	C	.	C
Thalictrum	?	G	G	C	.	T	A	.	C	.	G	.	C	.	T	.	.	A	G	.	.	C	.	T	.	.	.	T	G	.
Trollius	C	?	T	T	.	.	G	.	T	C	C	C	T	.	.	.	.	.	A	.	.	A	.	A	.	.	.	C	.	T
Xanthorhiza	T	C	?	.	T	.	.	G	A	T	T	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	?	C
Hydrastis	?	?	T	.	C	.	A	A	C	.	C	.	C	.	.	G	.	.	.	C	C	.	.	.	.	.	A	.	G	.
Caulophyllum	.	G	G	.	.	.	G	.	.	.	.	.	T	.	.	.	T	.	.	A	.	C	A	.	G	T	.	.	C	.
Cocculus	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

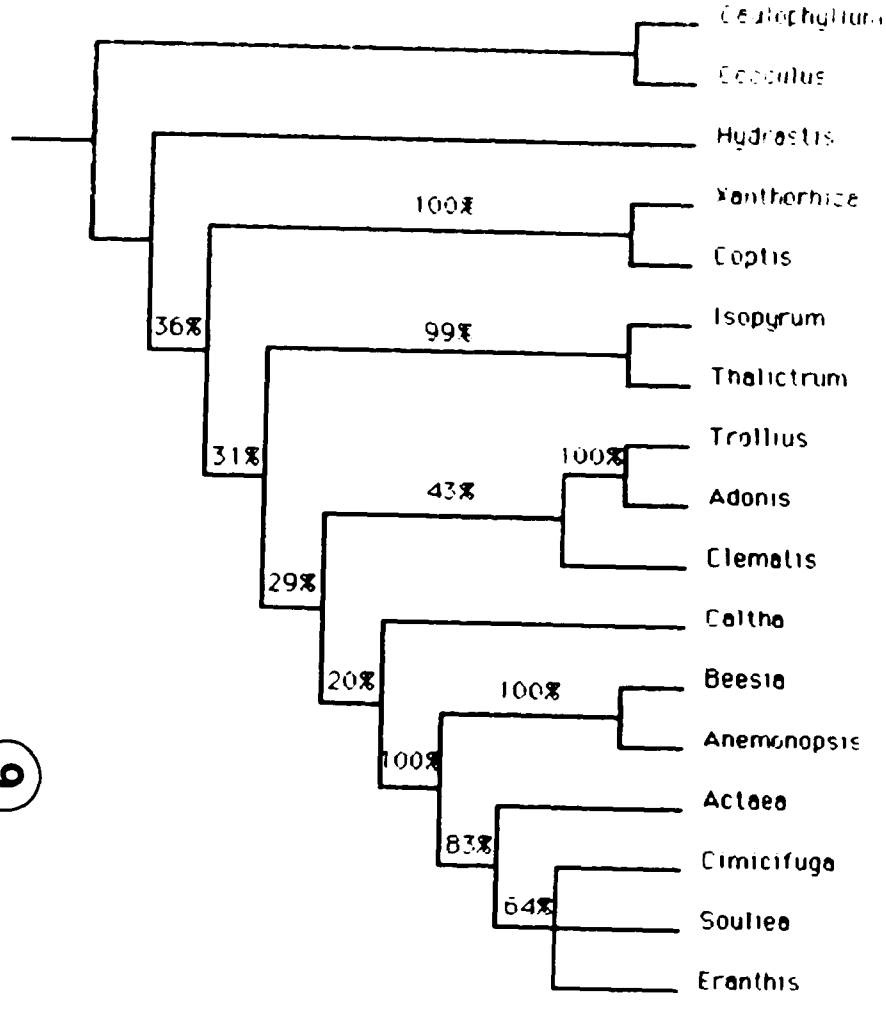
Table 11. continued.

					(1)								(1)								(1)					(1)				
	7	7	7	7	7	7	7	7	7	7	8	8	8	8	8	9	9	9	9	9	9	9	9	9	1	1	1	1	1
	5	5	5	6	7	8	8	9	9	9	0	1	1	1	5	0	0	0	2	4	5	5	6	7	9	0	0	1	2	2
	1	4	7	0	5	2	7	0	3	9	8	1	5	7	3	1	4	7	5	0	6	8	1	9	4	1	6	9	4	7
Actaea	A	G	A	A	T	T	G	G	G	C	T	C	G	T	C	A	C	C	G	T	G	T	T	G	G	T	C	C	T	T
Adonis	.	A	.	.	.	.	.	.	A	.	.	T	.	.	.	G	T	T	.	.	.	.	.	.	.	.	T	.	.	.
Anemonopsis	.	G	.	.	.	.	A	.	G	.	.	C	.	.	.	A	.	C	C	.	T	.	.	.	.	.	.	.	.	.
Beesia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Caltha	.	.	.	.	.	.	G	.	A	.	.	T	.	.	.	.	.	.	G	C	.	.	C	.	.	.	C	.	.	.
Cimicifuga	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	C	.	.	T	.	.	T	.	.	.	.	.	.	.
Clematis	.	.	.	.	C	C	.	.	A	T	C	.	.	.	.	G	T	.	.	.	G	C	.	.	.	C	.	.	.	.
Coptis	G	.	G	G	T	T	.	.	.	C	T	.	T	.	T	A	.	.	.	.	.	T	G	A	.	.	.	.	C	C
Eranthis	A	.	A	A	.	.	.	.	G	.	.	.	G	.	C	.	C	T	.	.	T	.	T	G	.	T	.	.	T	T
Isopyrum	.	.	.	?	C	C	.	T	A	.	.	.	.	.	.	.	T	C	.	.	.	.	.	A	.	.	.	.	.	.
Soulïea	.	.	.	A	T	T	.	G	G	.	.	.	.	.	.	.	C	.	.	.	.	.	.	G	.	.	.	.	.	.
Thalictrum	.	.	.	.	C	.	A	T	A	.	C	.	.	.	.	.	T	.	.	.	.	C	.	A	.	.	.	.	.	.
Trollius	.	A	C	.	T	.	G	G	.	T	T	T	.	.	.	G	.	T	.	.	G	T	.	G	.	.	.	.	.	.
Xanthorhiza	G	G	G	G	.	.	?	.	.	C	.	C	T	.	T	A	.	C	.	.	T	.	G	A	.	.	T	.	.	.
Hydrastis	A	.	A	C	.	.	G	.	.	.	C	.	G	.	C	G	.	.	.	C	G	.	T	.	.	.	C	.	.	.
Caulophyllum	.	.	.	A	.	A	.	.	.	T	T	.	.	C	.	.	.	.	.	.	.	.	.	.	T	.	.	T	.	.
Cocculus	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	.	.	.	T	T	.	C	G	.	C	.	.	.	.

Table 11. continued.

	(1)(2)									(1)																				(2)
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	3	3	3
	3	3	6	6	6	7	8	8	9	9	0	0	1	2	3	4	5	5	8	8	1	4	5	5	7	7	7	0	1	3
	1	2	3	7	9	5	1	7	3	7	2	5	1	3	8	4	0	9	0	6	6	3	2	5	3	6	9	9	2	2
Actaea	T	T	C	A	T	C	C	A	C	C	T	T	A	C	C	G	G	G	C	A	A	C	C	T	G	G	T	C	G	T
Adonis	.	A	.	.	C	T	T	G	T	.	C	.	.	.	T	.	.	.	.	.	.	.	.	C	.	A	.	.	T	.
Anemonopsis	.	T	.	.	T	C	C	A	C	.	.	.	.	.	C	.	.	.	.	.	.	.	T	T	.	.	.	.	.	.
Beesia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
Caltha	.	.	.	.	.	T	.	.	T	.	.	.	.	.	T	.	.	.	T	.	.	.	C	.	.	.	.	.	.	.
Cimicifuga	.	.	.	.	.	C	.	G	C	.	T	.	.	.	C	.	A	G	C	.	.	.	.	.	.	G	.	.	.	.
Clematis	.	.	.	.	.	T	.	A	.	T	C	.	G	.	T	A	G	.	.	.	.	.	.	.	.	A	.	.	.	.
Coptis	.	.	.	.	.	.	A	.	T	C	T	.	A	.	.	G	.	.	T	.	.	.	.	.	.	.	.	.	C	.
Eranthis	.	.	.	.	.	C	C	.	C	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	.	G	.	.	T	.
Isopyrum	.	.	.	.	.	T	.	.	T	.	C	.	G	.	T	A	A	.	.	.	G	.	.	.	A	A	C	.	C	.
Souliea	.	.	.	.	.	C	.	.	C	.	T	.	A	.	C	G	G	.	.	.	A	.	.	.	G	G	T	.	T	.
Thalictrum	.	.	.	.	.	T	.	.	T	.	.	.	.	.	T	A	.	.	.	C	G	.	T	.	A	A	C	.	.	.
Trollius	.	A	.	.	C	.	.	G	.	.	C	.	.	.	.	G	.	.	.	A	A	.	C	C	G	.	T	.	.	.
Xanthorhiza	.	T	.	.	T	.	.	.	.	.	.	.	.	.	.	?	.	.	.	?	?	.	T	T	A	.	C	.	.	.
Hydrastis	.	.	T	.	.	.	T	C	A	T	T	.	C	.	A	G	.	.	T	C	A	.	C	.	G	.	T	T	.	C
Caulophyllum	G	.	.	G	C	.	C	A	G	C	C	G	A	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
Cocculus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	G	.	.	.	.	C	C	.

Figure 9. Strict consensus tree of three equally parsimonious trees based on 247 variable nucleotide positions of rbcL, 120 of which are phylogenetically informative (Table 11). Numbers above nodes indicate the number of times that a monophyletic group occured in 500 bootstrap replicates.


Cimicifuga
Coptis
Hydrastis
Xanthorhiza
Coptis
Isopyrum
Thalictrum
Trollius
Adonis
Clematis
Caltha
Beesia
Anemonopsis
Actaea
Cimicifuga
Souliea
Eranthis
100%
36%
99%
31%
100%
43%
29%
20%
100%
100%
83%
64%

6

Table 12. The ratio of DNA contents.

	Total	A	T	G	C
Actaea	1328	358	370	332	268
Adonis	1318	362	375	326	255
Anemonopsis	1325	361	374	324	266
Beesia	1326	359	375	324	268
Caltha	1328	364	375	322	267
Cimicifuga	1322	357	370	327	268
Clematis	1333	360	372	330	271
Coptis	1323	357	372	330	264
Eranthis	1333	362	376	325	270
Isopyrum	1322	357	373	326	266
Souliea	1324	360	369	326	269
Thalictrum	1324	360	374	325	265
Trollius	1324	361	370	328	265
Xanthorhiza	1322	356	369	330	267
Hydrastis	1323	361	371	324	267
Caulophyllum	1323	358	369	335	261

Table 13. Data matrix used in Hennig 86.
Numbers are 0 = A, 1 = T, 2 = C, and 3 = G respectively.
Missing information is indicated by "?".

Caulophyllum (outgroup)
030311321132310212130033103322002122211210211111131013222??323232121232213130231123331320201012133110330002212030200313013202233203002012033011320112211301231321111132331232111131103113212032323111232303132020203210313012233132222

Actaea
022011221130322222133010103312002232211010212112231020327?102??321?11232223130031123231310102203123330023211030010023013202333203002012133321231011221130223132111313133313221111110321012202231112223230313202020322021331233313231

Adonis
032011321330302021103032103311002232221010212112231020???????2??32?2111320131001312101322310?12103120330023211030010023313202033100007012133021131011221133113132111313133313121111012321021131232112123230313202020322022301213313221

Anemonopsis
032011321130322222133010103312002232211010212112231020323?????????2112322231300311232313231010112031233300232110300100230132020332030020121033212310112211201231321111313333131211111103210122022321122232303102020203220113012133132211

Beesia
032011321130322222133010133312002232211010212112231020?32?21?????22112322231300311232313231210112031233300232110300100230132020332030020121033212310112211301231321111313333131221111103210122022321122231303102020203220113012133132211

Caltha
032011301130022222130010103312001232212010312112231020323210212311211231213130231123231010221021?1203300232?1030210023023207?033203002012133021131012221230123132111320233313221111120321011201202112123230313102020322021301213313121

Cimicifuga
032011321130022222133010103312002232213010212112131020????1???????2112322231300311232313231010122031233300232210302100230132020332030020121333212310112211302231321111313333132211111103210122322311122232030132020203220213312133132211

Clematis
332013301130300222133110103312302232122001222212131020323110232321211202213130231123231010211001103320232110300100230132020032030020222330122313111211331231312011132313323221111111032101120213211212023031320202002202130121331322111

Table 13. continued.

Coptis
0323213011200222222300133333120022332220102121122311207?2?2????????2111322131330321
1323132310101210312033000321103001002321320233323333201213302121101121113012313211
1313303320221221110321011001231112113230313102020320213012233132 21

Eranthis
0320113011300222221330101033120021321110102121222310203030102323211212322231300211
2323131310101220232323002321133001102301320203320300201213332123101122113021232211
1113133313221111110022012202231112223230313202020322021331113313221

Isopyrum
0323113011303222221330301333120022320220102121122310203232132?????212232213130201 1
3323132310102210312033032311102201002301 32?10?31030?2021231021231011221130123132 11
1113103313221111110321011201232112120230013202023322021002223313221

Soulieа
0320113211300222221330101033120022322110102121122310207?32?????2?2112322031300311
2323132310101220312333002321103001002201220203220300201213332123102122213022313211
1113133313221111110321012202231122223230313202020312021331212213221

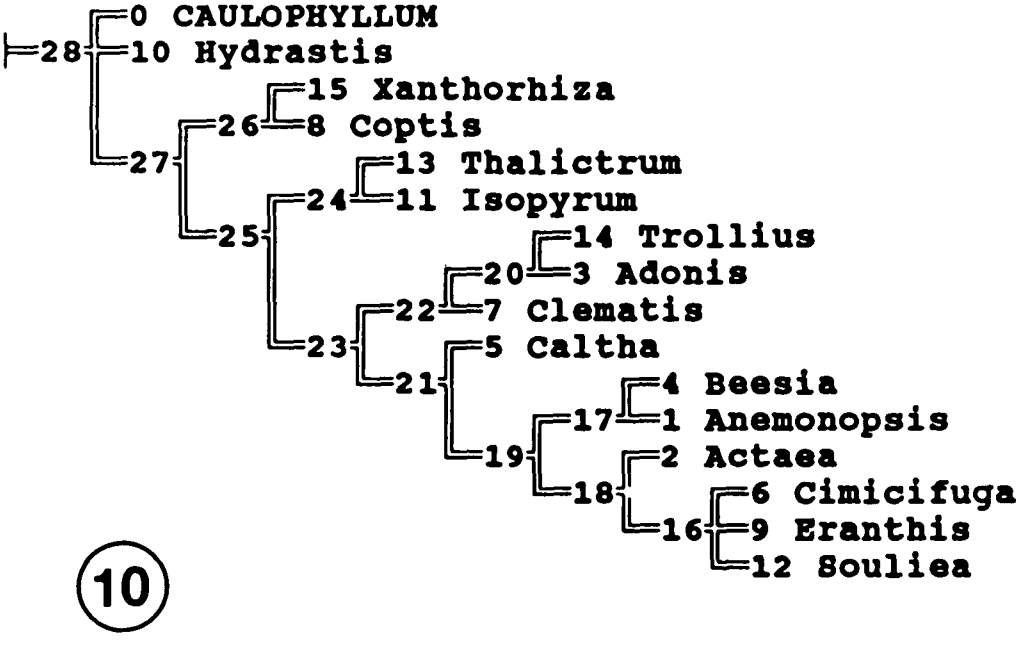
Thalictrum
0323113011303102221330301033120322322201021211223102 0?????3???23??212232210130211 1
3323132310102210312033032311030211023013202333203002022101022231011221130123132 11
1123103313221111110321011201231112120230313220203322011002213313221

Trollius
0320113013303020221030201023120022322220102121122010203232?????1??211232213130131 2
2323232310201210312033002321103001002301320233310020231213302113101122113311313211
1313133313121111010321021231232112123230313202020322022301213313221

Xanthorhiza
0323213231300222222300103333120022322220102121122321203221023237??211132213133031 1
1323132310101210312033000321103001002321303 2?3323333?0121?3021211 01?21113012313211
1113303323221221110321011001231112 1??2303132?202?32201100221333222 1

Hydrastis
0323113011303222221300101032220022322220102121122310237??1????1??211232210230231 12
3201323101022133120300023212030010323203023012133022231011221133123132112312100313
2211111103110112011321121232303031220203223213011133132 22

Figure 10. Nelsen tree generated by Hennig 86 using the data matrix shown in Table 13.


0 CAULOPHYLLUM
28
10 Hydrastis
15 Xanthorhiza
26
8 Coptis
27
13 Thalictrum
24
11 Isopyrum
25
14 Trollius
20
3 Adonis
22
7 Clematis
23
5 Caltha
21
4 Beesia
17
1 Anemonopsis
19
2 Actaea
18
6 Cimicifuga
16
9 Eranthis
12 Souliea

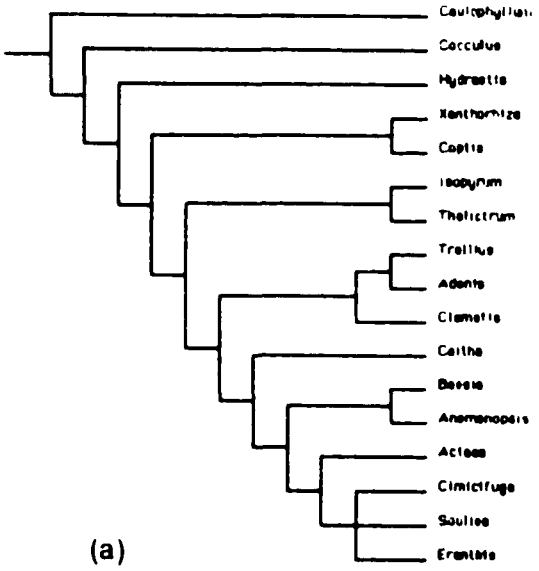
10

Figure 11. The resultant of three different trees by using different outgroups.

(a) Caulophyllum (Berberidaceae) and Cocculus (Menispermaceae) are used together as a outgroup.

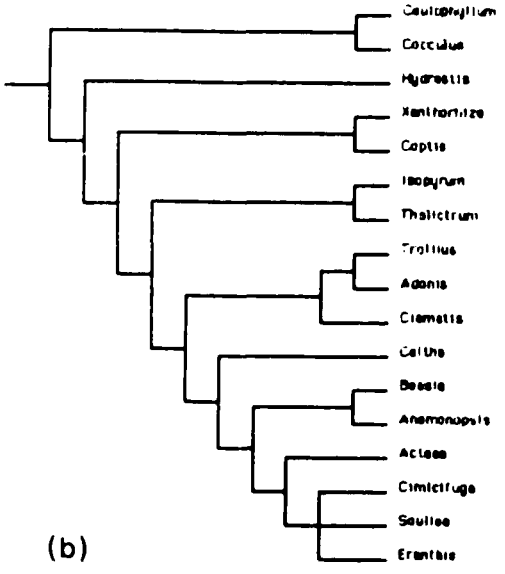
(b) Caulophyllum is used as a outgroup.

(c) Cocculus is used as a outgroup.

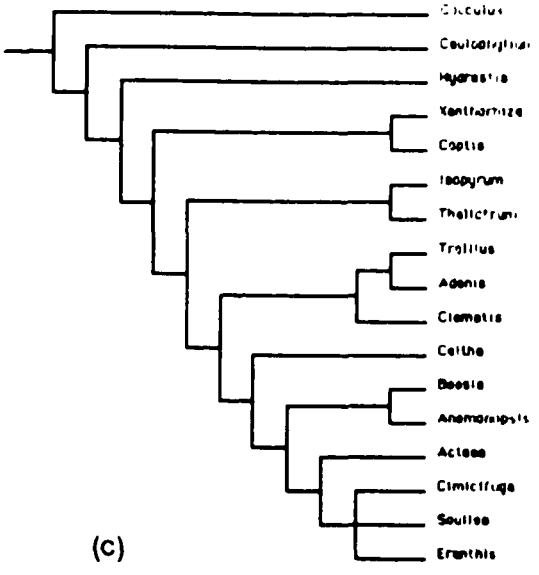

Cocculus
Hydrastis
Xanthorhiza
Coptis
Isopyrum
Thalictrum
Trollius
Adonis
Clematis
Caltha
Beesia
Anemonopsis
Actaea
Cimicifuga
Souliea
Eranthis

(a)

11


Cocculus
Hydrastis
Xanthorhiza
Coptis
Isopyrum
Thalictrum
Trollius
Adonis
Clematis
Caltha
Beesia
Anemonopsis
Actaea
Cimicifuga
Souliea
Eranthis

(b)


Cocculus
Hydrastis
Xanthorhiza
Coptis
Isopyrum
Thalictrum
Trollius
Adonis
Clematis
Caltha
Beesia
Anemonopsis
Actaea
Cimicifuga
Souliea
Eranthis

(c)

Table 14. Eleven informative amino acid positions with characters numbered by codon position. Abbreviations for 20 amino acids are:

A=Alanine, R= Arginine, N=Asparagine, D=Aspartic acid, C=Cysteine, Q=Glutamine, E=Glutamic acid, G=Glycine, H=Histidine, I=Isoleucine, L=Leucine, K=Lysine, M=Methionine, F=Phenylalanine, P=Proline, S=Serine, T=Threonine, W=Tryptophan, Y=Tyrosine, and V=Valine respectively.

	14	21	22	216	217	242	272	300	319	344	444
Actaea	T	A	P	I	Y	M	A	I	A	F	V
Adonis	.	E	.	.	.	I	.	M	.	Y	L
Anemonopsis	.	A	.	.	F	.	.	I	S	F	.
Beesia	.	.	.	.	Y	.	.	.	.	.	.
Caltha	N	T	.	L	.	.	.	.	.	.	.
Cimicifuga	T	.	.	.	.	.	.	.	.	.	.
Clematis	N	E	T	I	.	.	.	M	A	.	.
Coptis	.	T	P	.	.	M	S	I	.	.	.
Eranthis	.	.	.	.	F	I	A	.	S	.	.
Isopyrum	.	A	.	.	Y	.	.	.	.	.	.
Souliea	T	T	.	.	.	.	.	.	.	.	.
Thalictrum	N	V	T	L	F	M	.	.	.	.	.
Trollius	.	E	P	I	Y	.	.	M	A	Y	.
Xanthorhiza	T	T	.	.	.	?	S	I	S	F	.
Hydrastis	N	A	.	.	.	M	A	M	A	.	P
Caulophyllum	T	V	T	L	N	I	.	I	S	V	.

CHAPTER VIII. MOLECULAR DATA VS. MORPHOLOGICAL DATA

Introduction

Molecular data, particularly from cpDNA, is being used more and more frequently in systematic and phylogenetic studies of plants. When a method becomes commonly accepted, controversial issues about how to handle the molecular data was raised and discussed among the workers of molecular systematists and systematists working with morphological and other data (Miyamoto, 1985; Cracraft & Mindell, 1989; Hillis, 1987; Systma, 1990; Donoghue & Sanderson, 1991; Doyle, 1992; Caputo et al., 1992). There are two schools of thought: one in favor of combining both morphological and molecular data in reconstructing phylogeny (Donoghue & Sanderson, 1992); the second view is that phylogeny can be obtained solely on the basis of molecular data, and that morphological data can be set safely aside at the outset of an analysis and mapped onto the molecular phylogeny later (Gould, 1985; Sibley & Ahlquist, 1987). The latter view seems to be popular and widespread in botanical circles. For example, Sytsma et al. (1991) specifically recommend a "two-step process" in which trees based only on molecular data are used to interpret the evolution of morphological characters.

Before discussing this issue, we have to keep in mind that the product of phylogenetic reconstruction from molecular data is a 'gene tree' not necessarily a 'species tree'. Thus, no matter how well-resolved and strongly-supported a cpDNA phylogeny, it is only by extension a hypothesis of relationships among the plants bearing those chloroplast genomes. (Doyle, 1992). To add to this, Doyle (1992) mentions Nei's statement (1987) about estimation of species trees from gene trees, which are not necessarily synonymous.

Until now there are few careful morphological and molecular cladistic studies of the same groups of plants. In this study, I analyze morphological data and molecular data with a third method proposed by Caputo et al. (1992). This method is to fit the most parsimonious topology for a given data set onto another data set, and to compute the increase in step number. To accomplish this, I first generated a matrix for morphological characters and make comparisons with previous non-molecular studies.

Data sets

Although a various body of data has accumulated from studies of embryology, palynology, cytology. anatomy, and serology, phylogenetical studies of Ranunculaceae traditionally has been based primarily upon a set of morphological data. In the present study, I attempt to integrate these data to improve a view of the group. I put a character-state table based on 14 characters for 16 taxa. However, a fully resolved topology for n taxa requires at least n - 1

binary characters (Caputo et al., 1992), however I could not find more good conservative characters because, as mentioned previously, many useful characters such as anatomical, embryological, etc. are still missing for Asian, particularly Chinese, genera. Then, I incorporated several epidermal microcharacters surveyed by Hoot (1991) to have adequate numbers of character for cladistic analysis.

Studies of the epidermis of the Ranunculaceae have been limited chiefly to such foliar characters as stomates and trichomes. Hoot (1985) undertook a comprehensive survey of epidermal microcharacters of 20 genera in the family. In the following study, she surveyed 50 microcharacters of which 12 characters were chosen for the cladistic analyses. Hoot (1991) conducted phylogenetical study of the Ranunculaceae based on 12 epidermal microcharacters and 10 macromorphological characters. Because I have taken morphological characters from literatures, I combined my 14 characters with Hoot's eight informative epidermal microcharacters for genera common to both studies. I put a character-state table (Table 16) based on a character table (Table 15). The original 12 epidermal microcharacters are listed in Table 17. Here, I need to make a note that I am looking at genera which overlap between Hoot's work and my work which means that the three Asian genera are not included in the morphological analysis and also not included when making the comparison between a morphological tree and a molecular tree. Combined data sets are

again analysed using Wagner parsimony (branch and bound option) and produced one tree with 66 steps (Figure 12). This cladogram shows Hydrastis as the first branch of the Ranunculaceae and the rest of genera are in two clades one corresponding to a small chromosome group and the other corresponding to large chromosome.

Comparison of tree generated from current study and. Hoot's tree.

Although only 12 genera are in common in both Hoot's study and the present study, few congruent clades and some incongruencies are found. For example, a clade for Cimicifuga/Actaea and a clade for Coptis/Xanthorhiza are supported in both studies. In Hoot's study, the position of Caltha and Trollius is unresolved, however, they are in a same clade supporting the traditional view for these taxa. In contrast, the two taxa appeared in different clades in the present study. The latter view was suggested in a study of RFLPs of cpDNA by Johansen & Jensen (1991) and this view was again shown in a molecular data tree which I will discuss further in next chapter. The remainder of the genera are unresolved in both studies.

Comparison of molecular tree vs. morphology tree.

To make a comparison, a molecular tree without three Asian genera was generated using Wagner parsimony (branch and bound option) and produced one tree with 219 steps (Figure 13). The

combined morphology data generated a single tree (Figure 12) and was used as the morphology tree. Now using a method proposed by Caputo et al. (1992), I could calculate a "congruency index" (C) for both topologies, calculation equation defined as:

$$C_1 = 1_1/1_{1f} \text{ and } C_2 = 1_2/1_{2f}$$

1_1 = length of the most parsimonious topology for matrix a

1_{1f} = length of topology B fitted to matrix a

1_2 = length of the most parsimonious topology for matrix b

1_{2f} = length of topology A fitted to matrix b

In my study, a is the molecular data and b is the combined morphological data. Then the information is as follows:

1_1 = 219 steps

1_{1f} = 262 steps

1_2 = 66 steps

1_{2f} = 74 steps

Therefore, C_1 = 219/262 = 0.836 and C_2 = 66/74 = 0.892. From these calculation, it shows that 1_{2f} of the molecular tree or, what is the same 1_1, is more parsimonious topology and the true topology for a study group because a lower number of homoplasies is required to accept their topologies. Another explanation would be taking molecular data to fit the morphological topology causing an increase of 43 steps (from 219 to 262 steps) or a 20 % increase in homoplasy whereas taking morphological data to fit molecular topology causing an increase of 8 steps (from 66 to 74 steps) or a 12 % increase in homoplasy (Figure 14). Therefore, the molecular topology requires a

lower number of homoplasies to accept morphological data than does the morphological topology to accept the molecular data. Now I can accept the topology based on molecular data in assessing and reconstructing the phylogeny of study group.

Table 15. Characters and character states used in the cladistic analysis. The numbers with asterisles are micromorphological characters taken from Hoot (1991) used in the combined analyses. Literature sources of data used in the analysis are indicated at the end of table.

1. Number of carpel: 0 = one, 1 = 2 to 5, 2 = more than 6.
2. Chromosome size: 0 = large, 1 = small.
3. Chromosome base number: 0 = 10 or higher (n), 1 = 8, 2 = 7, 3 = 9.
4. Number of aperture: 0 = 0, 1 = 3, 2 = more than 4.
5. Type of aperture: 0 = none, 1 = colpate, 2 = polycolpate.
6. Inflorescence: 0 = determine, 1 = solitary.
7. Perianth form: 0 = sepals petaloid, 1 = petals petaloid.
8. Staminodia (nectaries): 0 = absent, 1 = present, 2 = flabelliform.
9. Ovule number: 0 = few (1-4), 1 = several (4 or more).
10. Bundle sheaths: 0 = absent, 1 = A-type, 2 = R-type.
11. Vessel perforation plates: 0 = scalariform, 1 = simple.
12. Xylem is V-shaped: 0 = present, 1 = absent.
13. Vasculature: 0 = dorsiventral type, 1 = radial type, 2 = radial scattered type.
14. Fruit type: 0 = follicle, 1 = achene, 2 = berry.

Table 15. continued.

Additional micromorphological data taken from Hoot (1991) used in the cladistic analysis:

15*. Papillae on the periclinal cell walls of leaves: 0 = absent, 1 = present.

16*. Surface of periclinal cell walls: 0 = smooth, 1 = striated.

17*. Stomata on adaxial leaf surface: 0 = absent, 1 = present.

18*. Trichome type 1: 0 = present, 1 = absent.

19*. Trichome type 3: 0 = absent, 1 = present.

20*. Trichome type 4: 0 = absent, 1 present.

21*. Micro-ornamentation on trichome type 1: 0 = smooth, 1 = micropapillate.

22*. Stomatal length: 0 = < 35 μm, 1 = > 35 μm.

Selected references:

Morphology (Kumazawa, 1932a, 1938b; Tamura, 1963)
Embryology (Davis, 1966; Tobe, 1974a, 1974b, 1989; Tobe & Keating, 1985; Jalan, 1963; Kumazawa, 1938a).
Anatomy (Tamura, 1962a, 1962b; Kumazawa, 1932b)
Cytology (Langlet, 1928, 1931; Gregory, 1941; Kurita, 1957a, 1957b, 1958, 1961; Kawano & Ihara, 1967).
Palynology (Kumazawa, 1931; Erdtman, 1952; Meacham, 1981; Nowicke & Skvarla, 1981, 1982).

Table 16. Matrix of combining eight of microcharacters with astoris (Hoot, 1991) and fourteen of macrocharacters shown in Table 15. Here, only Caulophyllum (Berberidaceae) is used as an outgroup.

	Character number																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15*	16*	17*
Actaea	0	0	1	1	1	0	1	0	1	1	1	0	2	0	0	1	0
Anemonopsis	1	0	1	1	1	0	1	1	1	1	1	0	2	1	?	?	?
Beesia	0	0	1	1	1	0	0	0	1	1	1	0	1	1	?	?	?
Souliea	0&1	0	1	0	0	0	1	0	1	?	1	0	?	1	?	?	?
Cimicifuga	0	0	1	1	1	0	1	1	1	1	1	0	2	1	0	1	0
Caltha	2	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	1
Trollius	1	0	1	1	1	0	0	1	1	1	1	0	1	1	0	1	0
Adonis	2	0	0	1	1	1	1	0	0	0	1	0	0	2	0	1	0&1
Eranthis	1	0	1	1	1	1	0	1	1	0	1	0	1	1	0	1	0
Clematis	2	0	1	1	1	0	0	0	0	1	1	0	1	2	0	0	0&1
Isopyrum	2	1	2	1	1	0	1	1	1	0	1	0	1	1	0&1	0	0
Thalictrum	1	1	2	2	2	0	0	0	0	2	1	0	1	2	1	0	0
Coptis	2	1	3	2	2	0	1	1	1	2	1	0	1	1	0	0	0
Xanthorhiza	1	1	0	2	2	0	1	1	0	2	1	0	1	1	0	0	0
Hydrastis	2	0	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0
Outgroup	0	0	0	1	1	0	0	2	0	?	1	1	1	1	0	0	0

Table 16. continued.

	Character number				
	18*	19*	20*	21*	22*
Actaea	0	1	0	1	1
Anemonopsis	?	?	?	?	?
Beesia	?	?	?	?	?
Souliea	?	?	?	?	?
Cimicifuga	0	1	0	1	1
Caltha	1	1	0	0	1
Trollius	1	1	0	0	1
Adonis	0	1	0	0	1
Eranthis	0&1	1	0	0	1
Clematis	0	1	0	0	1
Isopyrum	1	0	1	0	0
Thalictrum	1	0	1	0	0
Coptis	0	0	0	1	0
Xanthorhiza	0	0	0	1	0
Hydrastis	0	0	0	0	0
Outgroup	0	0	0	1	0

Table 17. The epidermal microcharacters used in Hoot's cladistic analyses (1991) of the Ranunculaceae. The numbers with astorisles are characters used in combined data sets for cladistic analysis.

---

1 * Papillae on the periclinal cell walls of leaves: 0 = absent, 1 = present.

2 * Surface of periclinal cell walls: 0 = smooth, 1 = striated.

3 * Stomata on adaxial leaf surface: 0 = absent, 1 = present.

4 * Trichome type 1: 0 = present, 1 = absent.

5. Trichome type 2: 0 = absent, 1 = present.

6 * Trichome type 3: 0 = absent, 1 = present.

7 * Trichome type 4: 0 = absent, 1 = present.

8. Trichome type 1 length: 0 = < 1 mm, 1 = > 1 mm.

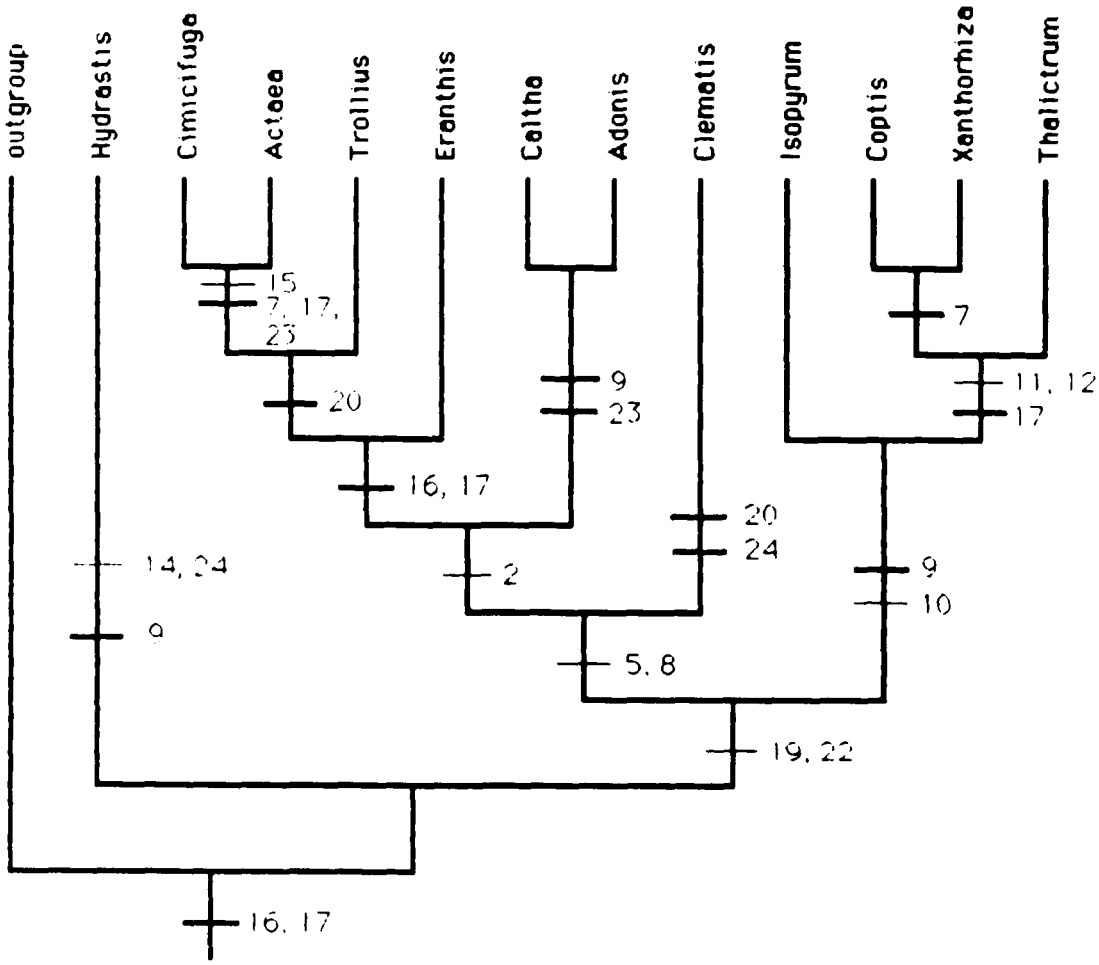
9*. Swollen base on trichome type 1: 0 = absent, 1 = present.

10. Raised rosette of cells surrounding base of trichome type1: 0 = absent, 1 = present.

11*.Micro-ornamentation on trichome type 1: 0 = smooth, 1 = micropapillate.

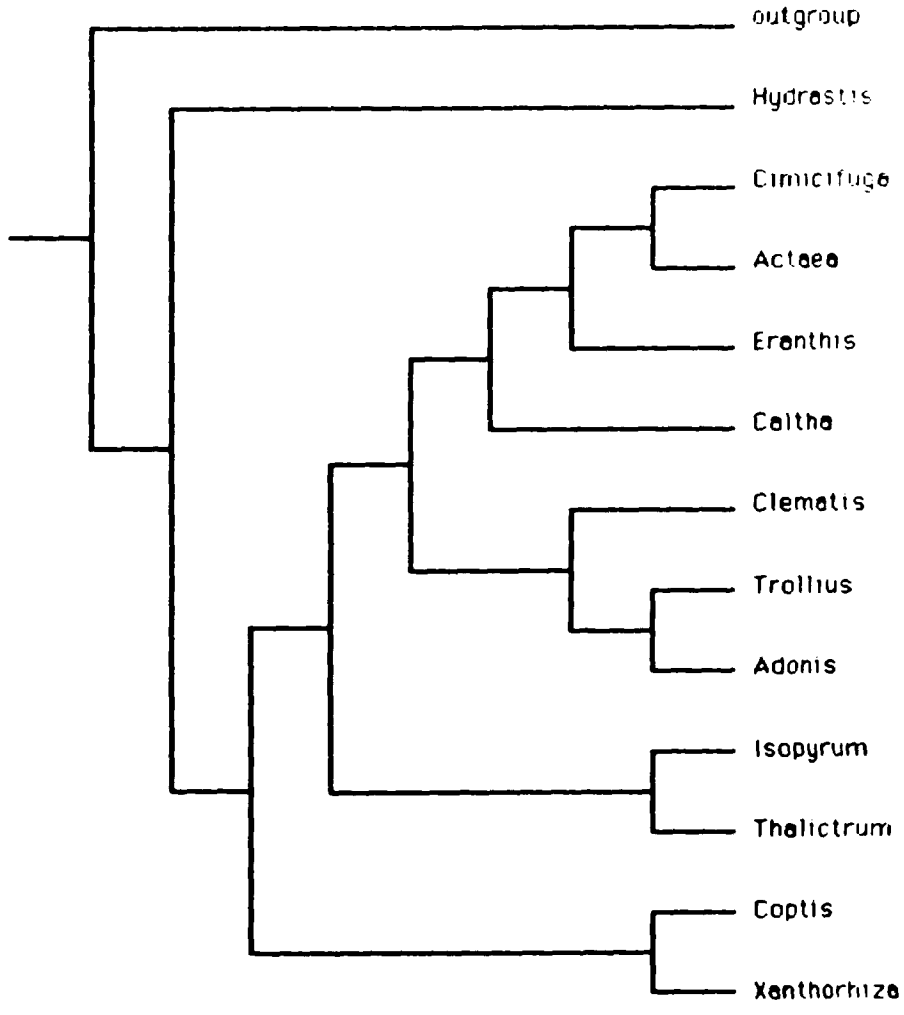
12*.Stomatal length: 0 = < 35 μm, 1 = > 35 μm.

Figure 12. A strict consensus tree of 66 steps from using combining both macro- and micromorphological characters. Three Asian genera are not included in this cladistic analysis.


outgroup
Hydrastis
Cimicifuga
Actaea
Trollius
Eranthis
Caltha
Adonis
Clematis
Isopyrum
Coptis
Xanthorhiza
Thalictrum
15
7, 17, 23
20
16, 17
9
23
14, 24
9
2
20
24
9
10
5, 8
19, 22
11, 12
17
7
16, 17

12

Figure 13. A strict consensus tree of 219 steps from using same rbcL sequences data from table 11 except the three Asian genera are excluded from this cladistic analysis.


outgroup
Hydrastis
Cimicifuga
Actaea
Eranthis
Caltha
Clematis
Trollius
Adonis
Isopyrum
Thalictrum
Coptis
Xanthorhiza

13

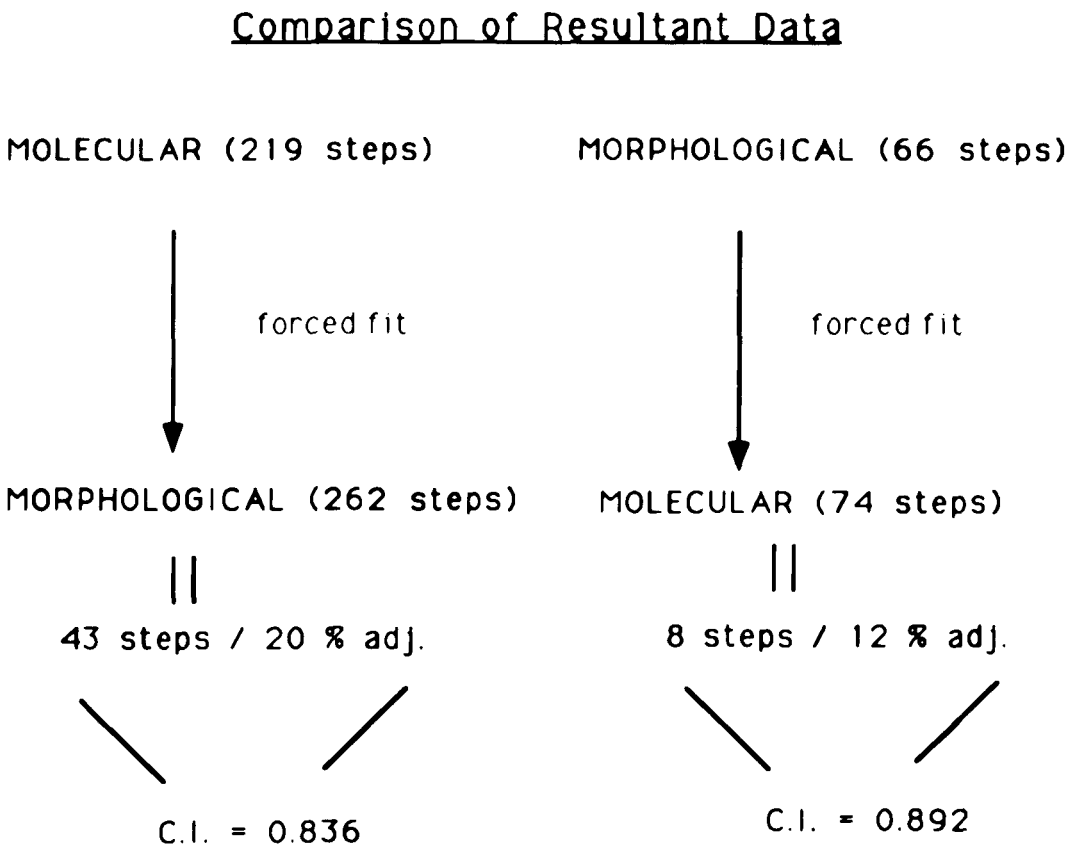


Figure 14 Comparison of "Congruency Index".

CHAPTER IX THE PHYLOGENY OF THE TRIBE CIMICIFUGEAE AND ALLIED GENERA IN THE RANUNCULACEAE.

The phylogenetic analysis of the tribe Cimicifugeae including three monotypic Asian genera and allied genera in the Ranunculaceae inferred from *rbc*L sequences was conducted for the first time. My goals were to obtain additional characters (*rbc*L sequence) for resolving incongruencies between phylogenies generated in previous morphological, cytological and serological studies and to perform phylogenetic analyses to provide a comprehensive and broadly based assessment of evolutionary relationships of the Cimicifugeae. Although in the previous chapter, molecular data proved to present more accurate picture of phylogeny, I feel morphological data are valuable characters for phylogenetic analyses of plants and both molecular and morphological data should be used in interpreting plant phylogeny. Since the consensus tree generated from molecular data using PAUP 3.0c and Hennig 86 was exactly the same, then I used Figure 9 of consensus tree and Figure 12 of consensus tree on the basis of morphology and other data to discuss the general phylogenetic implications of the Cimicifugeae and allied genera. Before an attempt to suggest a reconstructed phylogeny of the group, phylogenetic patterns seen in the previous chapter's cladistic analyses are noted.

1. The molecular and morphological topology support the position of Hydrastis to be the first branch of the Ranunculaceae, and also it is not a member of either outgroup.

2. Again, both topologies show two large clades; one is a group with large chromosomes and the other a group with small chromosomes.

3. Within the clade having small chromosomes, the molecular tree showed a resolution of two branches; Coptis/Xanthorhiza and Thalictrum/Isopyrum whereas in the morphology tree only the Coptis/Xanthorhiza clade is presented. According to Hoot (1991), the positions of Thalictrum and Isopyrum are unresolved, but at least they always appear in same clade as Coptis and Xanthorhiza.

4. In the clade having large chromosomes, when the three Asian genera are included in the analysis, the molecular tree has three new clades, Anemonopsis/Beesia, Cimicifuga/Souliea/Eranthis (as an unresolved trichotomy) and Trollius/Adonis. The basic topology did not change when the three Asian genera were excluded, except that Cimicifuga/Actaea are sister genera with Eranthis as the sister taxon to this clade.

5. The positions of Caltha and Trollius do not support the traditional grouping of these two genera as sister taxa because the two are in separate branches.

6. Clematis is always by itself and as in the analyses by Hoot (1991).

Phylogenetic implications.

The new information, *rbc*L sequences, provide some resolution of generic and tribal relationships in the Ranunculaceae. Even though *rbc*L data seems to show more accurate picture of phylogeny, it is best to consider the implications of the *rbc*L data in conjunction with other data sets in order to provide a balanced phylogenetic view.

Before discussing generic relationships in a tribe or in the family, the position of Hydrastis is mentioned because current analyses show that it does not belong in Ranunculaceae. Hydrastis, a monotypic genus occurring only in the temperate North America, has been placed historically in the Ranunculaceae or in the Berberidaceae and thus its systematic position has been controversial for many years. A previous serological study suggested that Hydrastis is closer to Ranunculaceae than to Berberidaceae (Jensen, 1968). Generally, Hydrastis is treated as a monotypic subfamily, Hydrastidioideae (Buchheim, 1964; Thorne, 1974, 1976; Cronquist, 1981) within the Ranunculaceae, but also has been considered to constitute the monotypic family, Hydrastidaceae. Lemesle (1948, 1950, 1955) first proposed an establishment of this monotypic family and this proposal was reestablished by Tobe and Keating (1985) after reevaluation of evidence from morphology, anatomy (including embryology), as well as from palynology, chemistry, and cytology.

In the present study, both morphological and molecular data support the systematic position of Hydrastis to be considered the first branch of the Ranunculaceae since the branch do not link to neither family of outgroup. In light of these evidences, the proposal of reestablishment of a monotypic family, Hydrastidaceae by Tobe and Keating (1985) is not supported in this study and this monotypic genus should remain in its own subfamily shown in some classifications (Cronquist, 1981, Takhtajan, 1987, Tamura, 1968).

As mentioned in previous chapter, there were not sufficient morphological data for the three Asian genera (Anemonopsis, Beesia, and Souliea), so their systematic positions will be discussed based on available molecular and cytological data. In the *rbc*L sequences data, Anemonopsis and Beesia are a sister group which occur with 100 % confidence level from 500 replicate bootstrap sampling. Tamura (1966) in his earlier classification, placed Beesia in the particular position in Trollieae and Anemonopsis in Cimicifugeae. Although the two genera are in separate tribe, but he suggested that both genera are in an intermediate situation between Trollieae and Cimicifugeae by the rhizome and the inflorescence. The inflorescence of Anemonopsis is an intermediate between determined panicle and raceme, and Beesia has a peculiar inflorencense racemiform in appearance where Cimicifugeaous genera have racemose inflorescences and Trollieaous genera does not have racemose inflorescences. In Tamura's most new classification (1990, 1991, 1992), he establishes a new subtribe

Beesiinae with Beesia along but kept Anemonopsis in Cimicifugeae. Karyotypes of both genera are available, but I do not find strong similarity in their karyotype except to say that both have x=8, large chromosomes, and larger subterminal chromosomes are satellited where normally the smallest subterminal chromosome is satellited in other genera of Cimicifugeae. The anatomical and embryological data are still not available, particularly for the Chinese genera, but the further examination of all these characters would help to support Beesia/Anemonopsis clade and removing Anemonopsis to Tamura's new subtribe Beesiinae with Beesia or even separate into a new tribe.

The third genus, Souliea often has been confused with Isopyrum or allied genera. Tamura (1966), without having the chromosomal data, considered that the genus is near to Cimicifuga rather than to Isopyrum because it has a racemose inflorescence. The cytological observation of Souliea in the present study shows that it has large chromosomes with basic number of 8, with the small satellites on the subterminal chromosomes supporting a position of Souliea near to Cimicifuga rather than to Isopyrum which has small chromosomes or to other genera in the tribe Cimicifugeae. This relationship was supported in the rbcL sequences data, except the clade with Souliea was unresolved by having Eranthis as third branch instead of Actaea, which has been traditionally considered to be the sister taxon of Cimicifuga. When the three Asian genera are excluded from both molecular and morphological analyses, there in

support a clade composed of Cimicifuga/Actaea. Karyotype analyses by Kurita (1957, 1959, 1961) concluded that in Ranunculaceae, satellites are not found in all genera with X=8 in the basic complement of which the two or more largest chromosomes are of median or near-median type, except for Cimicifuga and Actaea. Eranthis has been placed in tribe Helleboreae by Tamura (1966) and others which is another closely related group to the Cimicifugeae. This genus also has the basic number X=8 with characteristic satellited chromosomes. The two satellites in each cell differ in size from each other in that the larger satellite is three times as big as the small one. In fact the smaller satellited chromosome is very similar to that in Cimicifuga and Souliea. There were no other genera of Helleboreae in the molecular study to make a comparison for the position of Eranthis, but from the viewpoint of karyotype, Eranthis shares the characteristic feature of satellited chromosomes which are not found in other genera of Helleboreae and it is reasonable to accept it a trichotomous clade with Cimicifuga and Souliea. This view has been already mentioned in serological study of Jensen (1968) where Eranthis associated with an Actaea/Cimicifuga node and serologically separated from other Helleboreaous genera (Helleborus and Nigella) as shown in Figure 3.

The placement of Adonis with Trollius in molecular cladogram and Adonis with Caltha in morphological cladogram are both new alignments. The recognition of a close affinity between Caltha and Trollius is maintained by Langlet (1932), Gregory (1941), Janchen

(1949), and Tamura (1966). However, the serological study of Jensen (1968) emphasizes the dissimilarities between these two genera and places them in different subtribes within the tribe Caltheae. Recently, cladistic analysis of the Ranunculaceae have been published (Loconte & Estes, 1989; Hoot, 1991; Johansson & Jansen, 1991). In all of these studies, Caltha and Trollius have been included because the two genera have been considered to be the most likely hypothetical protoranunculacean, yet the cladistic relationships between the two remain unresolved. In the previous studies, the positions of the two were unresolved as a result of not having adequate related genera in the analysis. Although a study of restriction site comparisons (Johansson & Jansen, 1991) was conducted only with Trollius, Caltha, and several species of Ranunculus, it was evident from that study that Caltha and Trollius did not form a monophyletic group. Taking this evidence along with the results of the present study provides strong support that the traditional view of Caltha and Trollius as a monophyletic group is not correct. The new relationship of Trollius/Adonis is more strongly supported than that of Caltha/Adonis which was based mainly on serology (Figure 3). Caltha has long been considered to be the most primitive genus in the family by the lack of staminodia, and other unspecialized floral and fruit characters. Hoot (1991) mentioned that all macrocharacter states of Caltha are primitive, however microcharacters examined by her are apparently derived. Moreover, Caltha also has a more advanced karyotype than either

Trollius or Adonis. The position of Caltha in the Cimicifugeae is supported both serologically and cytologically.

Correlated small chromosome sizes were again confirmed in the present study. The clade of Coptis/Xanthorhiza is well supported both in molecular (100% in bootstrap) and morphological data (84% in bootstrap), where the clade of Isopyrum/Thalictrum is only formed by molecular data (99% in bootstrap). Both molecular and morphological data suggest a clade with all four genera. Cytological information clearly separate the genera into two groups which are characterized by chromosomes numbers X=9, Coptis/Xanthorhiza and X=7, Isopyrum/Thalictrum seemingly supporting the molecular results The view of Coptis/Xanthorhiza as a monophyletic group has been supported by all the previous studies and the present study reconfirmed this conclusion. On the other hand, the systematic position of this monophyletic group is controversial. Jensen and Greven (1984) hypothesized on the basis of serological data that Coptis/Xanthorhiza are closely related to Cimicifuga/Actaea and these taxa were basal to the family. The use of cladistic analyses with specified outgroups has given new insights into both the course and direction of evolution within this ancient family. The small chromosome group is hypothesized to be the most primitive in the Ranunculaceae (Hoot, 1991), a view that is neither supported by morphological cladogram based upon macro- and micromorphological characters nor by the molecular cladogram.

Re-examination of phylogenetic relationships among Cimicifugenous genera including the three monotypic Asian genera showed new alignments and some resolution. However, it is too early to propose a new classification for the Cimicifugeae because only molecular and cytological data are available for all genera and the other importat information such as anatomy and embryology is missing. The reconstruction of phylogenetic classification should be consider after all the morphological data become available.

When one is studying evolution, it may prove impossible to find a definitive answer to any particular case, but even a system of constraints based on real data would be an improvement over speculation (Nei, 1987). In any analyses, the utilization of all the available data is necessary to understand the study group but when data are difficult to interpret then, a new piece of information is needed to be examined. In the present study, rbcL sequence data is provided and shown to be valuable information on resolving incongruencies between phylogenies generated in previous morphological, cytological and serological studies in Ranunculaceae. Cronquist (1987) offered even stronger support for molecular systematics by suggesting that such an approach can be most useful by: 1) offering information that would help decide among different possible taxonomic arrangements, 2) encouraging the investigation of otherwise unconsidered taxonomic scenarios. There are many different methods that are used in analyzing data, and the use of cladistic analyses has given new insights. Cladistics continues to

be a stimulating, developing field, but much progress will be made when it is viewed more as a contest for analyzing and communicating about data than a set of principles for determining evolutionary history (Meacham, 1981). To continue this approach of studying evolution, further examination of all the genera including the monotypic genera which excluded from most of studies are necessary.

Appendix 1. Aligned sequence data (5'-> 3') for 17 taxa included in the cladistic analysis. Informative sites are denoted by a dot above the site. Missing information is indicated by "?".

Actaea	AAGTGTTGCCTTCAAAGCAGGTGTTAAAGATTACAAATTCACTTATTATACTCCTGAATA
Adonis	AAGTGTTGGCTTCAAAGCAGGTGTTAAAGATTACAAATTGACTTATTATACTCCGGAATA
Anemonopsis	AAGTGTTGGCTTCAAAGCAGGTGTTAAAGATTACAAATTGACTTATTATACTCCTGAATA
Beesia	AAGTGTTGGCTTCAAAGCAGGTGTTAAAGATTACAAATTGACTTATTATACTCCTGAATA
Caltha	AAGTGTTGGCTTCAAAGCAGGTGTTAAAGATTACAAATTGAATTATTATACTCCTGAATA
Cimicifuga	AAGTGTTGGCTTCAAAGCAGGTGTTAAAGATTACAAATTGACTTATTATACTCCTGAATA
Clematis	AGGTGTTGGCTTCAAAGCAGGTGTTAAAGAGTACAAATTGAATTATTATACTCCTGAATA
Coptis	AAGTGTTGGCTTCAAAGCGGGCGTTAAAGATTACAAATTGAATTATTATACTCCTCAATA
Eranthis	AAGTGTTGGCTTCAAAGCAGGTGTTAAAGATTACAAATTGAATTATTATACTCCTGAATA
Isopyrum	AAGTGTTGGCTTCAAAGCGGGTGTTAAAGATTACAAATTGAATTATTATACTCCTGAATA
Souliea	AAGTGTTGGCTTCAAAGCAGGTGTTAAAGATTACAAATTGACTTATTATACTCCTGAATA
Thalictrum	AAGTGTTGGCTTCAAAGCGGGTGTTAAAGATTACAAATTGAATTATTATACTCCTGAATA
Trollius	AAGTGTTGGCTTCAAAGCAGGTGTTAAAGATTACAAATTGAATTATTATACTCCGGAATA
Xanthorhiza	AAGTGTTGGCTTCAAAGCGGGCGTTAAAGATTACAAATTGACTTATTAGACTCCTGAATA
Hydrastis	AAGTGTTGGCTTCAAAGCGGGTGTTAAAGATTACAAATTGAATTATTATACTCCTGAATA
Caulophyllum	AAGTGTTGGATTCAAAGCGGGTGTTAAAGATTACAAATTGACTTATTATACTCCTGACTA
Cocculus	AAGTGTTGGATTCAAAGCGGGTGTTAAAGATTACAAATTGACTTATTATACTCCTGACTA

Appendix 1. continued.

Actaea	TGCACCCAAAGATACTGATACCTTGGCGGCATTCCGAGTAACTCCTCAACCTGGAGTTCC
Adonis	TGAACCAAAAGATACTGATACTTTAGCGGCATTCCGAGTAACTCCTCAACCGGGAGTTCC
Anemonopsis	TGCACCCAAAGATACTGATACCTTGGCGGCATTCCGAGTAACTCCTCAACCTGGAGTTCC
Beesia	TGCACCCAAAGATACTGATACCTTGGCGGCATTCCGAGTAACTCCTCAACCTGGAGTTCC
Caltha	TACACCCAAAGATACTGATACCTTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCC
Cimicifuga	TACACCCAAAGATACTGATACCTTGGCGGCATTCCGAGTAACTCCTCAACCTGGAGTTCC
Clematis	TGAAACCAAAGATACTGATACCTTGGCGGCATTCCGAGTATCTCCTCAACCTGGAGTTCC
Coptis	TACACCCAAAGATACTGATACCCTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCC
Eranthis	TACACCCAAAGATACTGATACCTTGGCGGCATTCCGAGTAACTCCTCAACCTGGAGTTCC
Isopyrum	TGCACCCAAAGATACTGATACCTTGGCGGCATTCCGAGTAACTCCTCAACCGGGAGTTCC
Souliea	TACACCCAAAGATACTGATACCTTGGCGGCATTCCGAGTAACTCCTCAACCTGGAGTTCC
Thalictrum	TGTAACCAAAGATACTGATACCTTGGCGGCATTCCGAGTAACTCCTCAACCGGGAGTTCC
Trollius	TGAACCAAAAGATACTGATACCTTAGCGGCATTCCGAGTAACTCCTCAACCCGGAGTTCC
Xanthorhiza	TACACCCAAAGATACTGATACCCTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCC
Hydrastis	TGCACCCAAAGATACTGATACCTTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCC
Caulophyllum	TGTAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACCGGGAGTTCC
Cocculus	TGTAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACCGGGAGTTCC

Actaea	ACCTGAAGAAGCAGGGGCTGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Adonis	CCCTGAAGAAGCAGGGGCTGCTGTAGCTGCTGAATCTTCTACAGGTACATGGACAACTGT
Anemonopsis	ACCTGAAGAAGCAGGGGCTGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Beesia	ACCTGAAGAAGCGGGGGCTGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Caltha	ACCTGAAGAAGCAGGGGCTGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Cimicifuga	ACCTGAAGAAGCAGGGGCTGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Clematis	ACCTGAAGAAGCAGGGGCTGCTGTAGCTGCCGAATCTTCTACGGGTACATGGACAACTGT
Coptis	GCCGGAAGAAGCGGGGGCTGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Eranthis	ACCTGAAGAAGCAGGGGCTGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Isopyrum	ACCTGAAGAAGCGGGGGCTGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Souliea	ACCTGAAGAAGCAGGGGCTGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Thalictrum	ACCTGAAGAAGCAGGGGCTGCTGTAGCTGCCGAATCTTCTACAGGTGCATGGACAACTGT
Trollius	ACCTGAAGAAGCACGGGCTGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Xanthorhiza	ACCGGAAGAAGCGGGGGCTGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Hydrastis	ACCTGAAGAAGCAGGCGCCGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Caulophyllum	GCCTGAAGAAGCAGGGGCCGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT
Cocculus	GCCTGAAGAAGCAGGGGCCGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACTGT

Appendix 1. continued.

Actaea	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATTGAGCC
Adonis	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATCGAGCC
Anemonopsis	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATTGAGCC
Beesia	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATTGAGCC
Caltha	GTGGACCGATGGACTTACTAGCCTTGATCGTTACAAAGGACGATGCTACCACATTGAGCC
Cimicifuga	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATTGAGCC
Clematis	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCATATCGAGCC
Coptis	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACGACATCGAGCC
Eranthis	GTGGACCGATGGACTTACCAGTCTTGATCGTTACAAAGGACGATGCTACCATATTGAGCC
Isopyrum	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCAAATCGAGCC
Souliea	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATTGAGCC
Thalictrum	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATCGAGCC
Trollius	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATCGAGCC
Xanthorhiza	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATCGAGCC
Hydrastis	GTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATCGAGCC
Caulophyllum	GTGGACCGATGGACTTACCAGTCTTGATCCTTACAAAGGACGATGCTACCACATTGAGCC
Cocculus	GTGGACCGATGGACTTACCAGTCTTGATCGTTACAAAGGACGATGCTACCACATTGAGCC
Actaea	TGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Adonis	TGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Anemonopsis	TGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Beesia	TGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Caltha	CGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCGTATCCTTTAGACCTTTTTGA
Cimicifuga	GGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Clematis	CGTTGCTGGAGAAGAAAATCAATATATTTGTTATGATGCCTACCCTTTAGACCTTTTCGA
Coptis	CGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Eranthis	TGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Isopyrum	CGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Souliea	TGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Thalictrum	CGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Trollius	CGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Xanthorhiza	CGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Hydrastis	CGTTGCTGGAGAAGAAAATCAATATATTTGTTATGTAGCCTATCCTTTAGACCTTTTTGA
Caulophyllum	TGTTGCTGGAGAAGACAATCAATATATTTGTTATGTAGCCTATCCTTTAGATCTTTTTGA
Cocculus	TGTTGCTGGAGAAGACAATCAATATATTTGTTATGTAGCCTATCCTTTAGATCTTTTTGA

Appendix 1. continued.

Actaea	AGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAAGC
Adonis	AGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAA?GTATTTGGGTTCAAA??
Anemonopsis	AGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAAGC
Beesia	AGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAA?G
Caltha	AGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAAGC
Cimicifuga	AGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTGGGTAATGTATTTGGGTTCAAA??
Clematis	AGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTGGGTAATGTATTTGGGTTCAAAGC
Coptis	AGAGGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTTTTTGGGTTCAAA?C
Eranthis	AGAAGGCTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAAGA
Isopyrum	AGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAAGC
Souliea	AGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAA??
Thalictrum	AGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAA??
Trollius	AGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTAGGTAATGTATTTGGGTTCAAAGC
Xanthorhiza	AGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGCTTTTGGGTTCAAAGC
Hydrastis	AGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAAG?
Caulophyllum	AGAAGGTTCTGTTACTAATATGTTTACTTCTATTGTGGGTAATGTATTTGGGTTTAAGCC
Cocculus	AGAAGGTTCTGTTACTAATATGTTTACTTCTATTGTGGGTAATGTATTTGGGTTTAAGGC

Actaea	??TAC??GCT?TACGTCTGGAGGATCTGCGAATCCCTGTTGCTTATGTTAAAACTTTCCA
Adonis	????C??GC?CTACGTTTGGAGGATCTGAGAATTCCTGTTGCTTATATTAAAACTTTCCA
Anemonopsis	GC?????????CTACGTCTGGAGGATCTGCGAATCCCTGTTGCTTATGTTAAAACTTTCCA
Beesia	C?CT?????CCTACGTCTGGAGGATCTGCGAATCCCTGTTGCTTATGTTAAAACTTTCCA
Caltha	GCTACTCGTTCTACGTCTGGAGGATTTGCGAATTCCTGTTGCTTATGTTAAAACTTTCCA
Cimicifuga	??T????????CTACGTCTGGAGGATCTGCGAATCCCTGTTGCTTATGTTAAAACTTTCCA
Clematis	GTTACGCGCTCTACGTCTGGAAGATCTGCGAATTCCTGTTGCTTATGTTAAAACTTTCCA
Coptis	?C?????????CTACGTTTGGAGGATCTGCGAATTCCTGTTGCTTATGTTAAAACTTTCCA
Eranthis	GATACGCGCTTCACGTCTGGAGGATCTGCGAATCCCTGTTGCTTATGTTAAAACTTTCCA
Isopyrum	GCTGC?????CTACGCCTGGAGGATCTGCGAATTCCTGTTGCTTATGTTAAAACTTTCCA
Souliea	GC??????C?CTACCTCTGGAGGATCTGCGAATACCTGTTGCTTATGTTAAAACTTTCCA
Thalictrum	???G??CG??CTACGCCTGGAGGATCTGCGAATTCCTATTGCTTATGTTAAAACTTTCCA
Trollius	GC?????T??CTACGTCTGGAGGATCTGCGAATTCCTGTTGCTTATGTTAAAACTTTCCA
Xanthorhiza	CTACGCG???CTACGTTTGGAGGATCTGCGAATTCCTGTTGCTTATGTTAAAACTTTCCA
Hydrastis	??T????T??CTACGTCTGGAGGATCTGCGAATTCCTACTGCTTATGTTAAAACTTTCCA
Caulophyllum	C??GCGCGCTCTACGTCTGGAGGATCTGCGAATTCCTGTTGCTTATGTTAAAACTTTCCA
Cocculus	GCTGCGCGCTCTACGTCTGGAGGATCTGCGAATTCCTGTTGCTTATGTTAAAACTTTCCA

Appendix 1. continued.

Actaea	ÀGGÀCCGCCTCATGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCT
Adonis	AGGTCCGCCTCACGGTATCCAAGTTGAGAGAGATAAATTAAATAAGTATGGCCCTCCCCT
Anemonopsis	AGGACCGCCTCATGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCT
Beesia	AGGACCGCCTCATGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCT
Caltha	AGGCCCGCCTCATGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCT
Cimicifuga	AGGACCGCCTCATGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCT
Clematis	AGGCCCGCCTCATGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCT
Coptis	GGGACCGCCCCATGGTATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCT
Eranthis	AGGACCCCCTCATGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCTT
Isopyrum	AGGCCCACCTCATGGGATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCT
Souliea	AGGACCGCCTCATGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCT
Thalictrum	AGGCCCTCCTCATGGGATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCT
Trollius	AGGTCCGCCTCACGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGCCGTCCCCT
Xanthorhiza	GGGACCGCCTCATGGTATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCT
Hydrastis	AGGCCCGCCTCATGGCATCCAAGTTGAGAGAGATAAATTGAACAAATATGGTCGTCCCCT
Caulophyllum	AGGCCCGCCTCATCGCATCCAAGTTGAGAGAGATAAATTGAAGAAGTATGGTCGTCCCCT
Cocculus	AGGACCGCCTCATGGC??
Actaea	ATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCTAAGAACTACGGCAGAGCGGT
Adonis	ATTGGGATGTACTATTAAACCAAAATTGGG??TATCTGCTAAGAACTACGGTAGAGCGGT
Anemonopsis	ATTGGGATGTACTATTAAACCAAAATTGGGATTATCTGCTAAGAACTATGGCAGAGCGGT
Beesia	ATTGGGATGTACTATTAAACCCAAATTGGGATTATCTGCTAAGAACTATGGCAGAGCGGT
Caltha	ATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCTAAGAACTACGGTAGAG??GT
Cimicifuga	ATTGGGATGTACTATTAAACCAAAATTGGGATTATCTGCTAAGAACTACGGCAGAGCGGT
Clematis	ATTGGGATGTACTATTAAACCAAAATTGGGCTTATCTGCTAAGAACTATGGTAGAGCAGT
Coptis	ATTGGGATGTACTATTAAACCAAAATTGGGATTATCTGCTAAGAACTACGGTAGAGCGGT
Eranthis	ATTGGGATGTACTATTAAACCAAAATTGGGATTATCTGCTAAGAACTACGGCAGAGCCGG
Isopyrum	AT?GGGATGTACTATTAAACCAAAATTGGGATTATCCGCTAAGAACTACGGTAGAGCGGT
Souliea	ATTGGGATGTACTATTAAACCAAAATTGGGATTATCTGCTAAGAACTACGGCAGAGCGGT
Thalictrum	ATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCTAAGAACTACGGTAGAGCGGT
Trollius	ATTGGGATGTACTATTAAACCAAAATCGGGATTATCTGCTAAGAACTACGGTAGAGCGGT
Xanthorhiza	ATTGGGATGTACTATTAAACCAAAATTGGGATTATCTGCTAAGAACTACGGTAGAGCGGT
Hydrastis	ATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCTAAGAACTACGGTAGGGCGGT
Caulophyllum	ATTAGGATGTACCATTAAACCAAAATTGGGATTATCTGCTAAGAACTACGGTAGGGCGGT
Cocculus	??????????????????AACCAAAATTGGGATTATCTGCTAAGAACTACGGTAGGGCGGT

Appendix 1. continued.

Actaea	TTATGAATGTCTCCGCGGTGGGCTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Adonis	TTATGAATGTCTCCGCGGTGGACTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Anemonopsis	TTATGAATGTCTCCGCGGTGGGCTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Beesia	TTATGAATGTCTCCGCGGTGGGCTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Caltha	TTATGAATGTCTCCGCGGTGGACTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Cimicifuga	TTATGAATGTCTCCGCGGTGGGCTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Clematis	TTATGAATGTCTCCGTGGTGGACTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCC
Coptis	TTATGAATGTCTCCGCGGTGGACTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Eranthis	TTATGAATGTCTCCGCGGTGGGCTTCATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Isopyrum	TTATGAATGTCTCCGCGGTGGACTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Souliea	TTATGAATGTCTCCGCGGTGGGCTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Thalictrum	TTATGAATGTCTCCGCGGTGGACTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Trollius	TTATGAATGTCTCCGCGGTGGACTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Xanthorhiza	TTATGAATGTCTCCGCGGTGGACTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Hydrastis	TTATGAATGTCTCCGCGGTGGACTTGATTTTACCAAGGATGATGAGAACGTAAACTCCCA
Caulophyllum	TTATGAATGTCTCCGTGGTGGACTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Cocculus	TTGTGAATGTCTCCGTGGTGGACTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCA
Actaea	ACCCTTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCTGAAGCAATTTATAAAGCACA
Adonis	ACCCTTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCTGAAGCAATTTATAAAGCACA
Anemonopsis	ACCCTTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCTGAAGCAATTTATAAAGCACA
Beesia	ACCCTTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCTGAAGCAATTTATAAAGCACA
Caltha	ACCCTTTATGCGTTGGAGAGACCGTTTCCTATTTT??GCTGAAGCACTTTATAAAGCACA
Cimicifuga	ACCCTTTATGCGTTGGAGAGACCGTTTCCTATTTTGCGCTGAAGCACTTTATAAAGCACA
Clematis	ACCCTTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCTGAAGCAATTTATAAAGCACA
Coptis	ACCATTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCTGAAGCAATTTATAAAGCACA
Eranthis	ACCCTTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCTGAGGCAATTTTTAAAGCACA
Isopyrum	GCCCTTTATGCGTTGGAGAGATCGTTTCCTATTTTGTGCTGAACCCATTTATAAAGCACA
Souliea	ACCCTTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCTGAAGCAATTTATAAAGCACA
Thalictrum	GCCCTTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCTGAAGCACTTTTTAAAGCACA
Trollius	ACCCTTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCTGAAGCAATTTATAAAGCACA
Xanthorhiza	ACCATTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCTGAAGCAATTTATAAAGCACA
Hydrastis	ACCCTTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCCGAAGCAATTTATAAAGCACA
Caulophyllum	ACCATTTATGCCTTGGAGAGACCGTTTCCTATTTTGTGCCGAAGCACTTAATAAAGCACA
Cocculus	ACCATTTATGCGTTGGAGAGACCGTTTCCTATTTTGTGCCGAAGCACTTAATAAAGCACA

Appendix 1. continued.

Actaea	AGCCGAAACAGGTGAAATCAAAGGACATTACTTGAATGCTACTGCGGGTACATGCGAAGA
Adonis	AGCCGAAACGGGTGAAATCAAAGGACATTACTTGAATGCTACTGCGGG?ACATGCGAAGA
Anemonopsis	AGCCGAAACAGGTGAAATCAAAGGACATTACTTGAATGCTACTGCGGGTACATGCGAAGA
Beesia	AGCCGAAACAGGTGAAATCAAAGGACATTACTTGAATGCTACTGCGGGTACATGCGAAGA
Caltha	AGCCGAAACAGGCGAAATCAAAGGACATTACTTGAATGCTACTGCGGGTACATG?GAAGA
Cimicifuga	AGCCGAAACAGGTGAAATCAAAGGACATTACTTGAATGCTACTGCGGGTACATGCGAAGA
Clematis	AGCCGAAACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCGGGTACATGCGAAGA
Coptis	AGCCGAAACCGGTGAAATCAAAGGACATTACTTGAATGCTACTGCGGGTACATGCGAAGA
Eranthis	AGCCGAAACAGGTGAAATCAAAGGACATTACTTGAATGCTACTGCGGGTACATGCGAAGA
Isopyrum	AGCCGAAACAGGTGAAATCAAAGGACATTATTTGAATGCTACTGCGGGTAC?TGTGAAGA
Souliea	AGCCCAAACAGGTCAAATCAAAGGACATTACTTGAATGCTACTGCGGGTACATGCGAAGA
Thalictrum	AGCCGAAACAGGTGAAATCAAAGGACATTATTTGAATGCTACTGCGGGTACATGCGAAGA
Trollius	AGCCGAAACAGGTGAAATCAAAGGACATTACTTGAATGCTACTGCGGGTACATGCGAAGA
Xanthorhiza	AGCCGAAACCGGTGAAATAAAAGGACATTACTTGAATGCTACTGCGGGTACGTGCGAAGA
Hydrastis	AGCCGAAACAGGTGAAATCAAAGGACATTACTTGAATGCTACTGCGGGTACATGCGAAGA
Caulophyllum	GGCTGAAACAGGTGAAATCAAAGGACATTACTTGAATGCTACTGCGGGTACATGCGAAGA
Cocculus	GGCTGAAACAGGTGAAATCAAAGGACATTACTTGAATGCTACTGCGGGTACATGCGAAGA

Actaea	AATGATGAAAAGGGCTGTATTTGCCAGAGAATTGGGAGTACCCATCGTAATGCATGACTA
Adonis	AATGATAAAAAGGGCTGTATTTGCTAGAGAATTAGGAGTACC?ATCGTAATGCATGACTA
Anemonopsis	AATGATAAAAAGGGCTGTATTTGCCAGAGAATTGGGAGTACCCATCGTAATGCATGACTA
Beesia	AATGATAAAAAGGGCTGTATTTGCCAGAGAATTGGGAGTACCCATCGTAATGCATGACTA
Caltha	AATGATAAAAAGGGCTGTATTTGCCAGAGAATTGGGAGTACCCATCGTAATGCATGACTA
Cimicifuga	AATGATAAAAAGGGCTGTATTTGCCAGAGAATTGGGAGTACCCATCGTAATGCATGACTA
Clematis	AATGATAAAAAGAGCTGTATTTGCCAGAGAATTGGGAGTACCCATCGTAATGCACGACTA
Coptis	AATGATGAAAAGGGCTGTATTTGCCAGAGAGTTGGGGGTGCCCATCGTAATGCATGACTA
Eranthis	AATGATAAAAAGGGCTGTATTTGCCAGAGAATTGGGAGTACCCATCGTAATGCATGACTA
Isopyrum	AATGATAAAAAG?GCTGTATTTGCTAGAGAATTGGGAGT?CCCATCGTAATGCACGATTA
Souliea	AATGATAAAAAGGCCTGTATTTGCCAGAGAATTGGGAGTACCCATCGTAATGCATGACTA
Thalictrum	AATGATGAAAAGGGCTGTATTTGCCAGAGAATTGGGAGTACCCATCGTAATGCACGACTA
Trollius	AATGATGAAAAGGGCTGTATTTGCTAGAGAATTAGGCGTACCCGTCGTAATGCATGACTA
Xanthorhiza	AATGAT?AAAAGGGCTGTATTTGCCAGAGAGTTGGGGGTGCC?ATCGTAATGCATGACTA
Hydrastis	AATGATGAAAAGGGCTGTATTTGCCAGAGAATTGGGAGTCCCGATCGTAATGCATGACTA
Caulophyllum	AATGATCAAAAGGGCTGTATTTGCCAGAGAATTGGGAGTACCCATCGTAATCCATGACTA
Cocculus	AATGATCAAAAGGGCTGTATTTGCCAGAGAATTGGGAGTACCCATCGTAATGCATGACTA

Appendix 1. continued.

Actaea	CTTAACGGGGGGGTTCACCGCAAATACTAGCTTGGCTCATTATTGCCGAGATAATGGTCT
Adonis	CTTAACGGGGGGATTCACCGCAAATACTAGTTTGGCTCATTATTGCCGAGATAATGGTCT
Anemonopsis	CTTAACAGGGGGGTTCACCGCAAATACTAGCTTGGCTCATTATTGCCGAGATAATGGTCT
Beesia	CTTAACAGGGGGGTTCACCGCAAATACTAGCTTGGCTCATTATTGCCGAGATAATGGTCT
Caltha	CTTAACGGGGGGATTCACCGCAAATACTAGTTTGGCTCATTATTGCCGAGATAATGGCCT
Cimicifuga	CTTAACGGGGGGGTTCACCGCAAATACTAGCTTGGCTCATTATTGCCGAGATAATGGTCT
Clematis	CCTAACGGGGGGATTCACTGCAAATACCAGCTTGGCTCATTATTGCCGGGATAATGGTTT
Coptis	CTTAACGGGGGGATTCACCGCAAATACTAGCTTGTCTCATTATTGCCGAGATAATGGTCT
Eranthis	CTTAACGGGGGGGTTCACCGCAAATACTAGCTTGGCTCATTATTGCCGAGATAATGGTCT
Isopyrum	CCTAACGGGTGGATTCACCGCAAATACTAGCTTGGCTCATTATTGCCGAGATAATGGTCT
Souliea	CTTAACGGGGGGGTTCACCGCAAATACTAGCTTGGCTCATTATTGCCGAGATAACGGTCT
Thalictrum	CTTAACAGGTGGATTCACCGCAAATACCAGCTTGGCTCATTATTGCCGAGATAATGGTCT
Trollius	CTTAACGGGGGGATTCACCGCAAATACTAGTTTGGCTCATTATTGCCGAGATAATGGTCT
Xanthorhiza	CTTAAC?GGGGGATTCACCGCAAAT?CTAGCTTGTCTCATTATTGCCGAGATAATGG?CT
Hydrastis	CTTAACGGGGGGATTCACCGCAAATACCAGCTTGGCTCATTATTGCCGAGATAATGGTCT
Caulophyllum	CATAACGGGGGGATTCACTGCAAATACTAGTTTGGCCCATTATTGCCGAGATAATGGTCT
Cocculus	CATAACGGGGGGATTCACTGCAAATACTAGTTTGGCCCATTATTGCCGAGATAATGGTCT

Actaea	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Adonis	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Anemonopsis	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATCGTAT
Beesia	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Caltha	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCACGGTAT
Cimicifuga	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Clematis	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Coptis	ACTTCTTCACATTCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Eranthis	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Isopyrum	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Souliea	ACTTCTTCACATCCACCGCGCAACGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Thalictrum	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Trollius	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Xanthorhiza	ACTTCTTCACATTCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Hydrastis	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Caulophyllum	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT
Cocculus	ACTTCTTCACATCCACCGCGCAATGCATGCAGTTATTGATAGACAGAAGAATCATGGTAT

Appendix 1. continued.

Actaea	ACACTTCCGTGTACTAGCTAAAGCGTTACGTATGTCTGGTGGAGATCATATTCACGCTGG
Adonis	GCATTTTCGTGTACTAGCTAAAGCGTTACGTATGTCTGGTGGAGATCATATTCACGCTGG
Anemonopsis	ACATTTCCGTGTACTAGCTAAAGCCTTACGTATGTCTGGTGGAGATCATATTCACTCTGG
Beesia	ACATTTCCGTGTACTAGCTAAAGCCTTACGTATGTCTGGTGGAGATCATATTCACTCTGG
Caltha	ACATTTCCGTGTACTAGCTAAAGCGTTACGTATGTCTGGCGGAGATCATATTCACTCTGG
Cimicifuga	ACACTTCCGTGTACTAGCTAAAGCGTTACGTATGTCTGGTGGAGATCATATTCACTCTGG
Clematis	GCATTTCCGTGTATTAGCTAAAGCGTCACGTATGTCAGGTGGAGATCATATTCACGCCGG
Coptis	ACATTTCCGTGTACTAGCTAAAGCGTTACGTATGTCTGGTGGAGATCATATTCACGCTGG
Eranthis	ACACTTTCCGCTACTAGCTAAAGCGTTACGTATGTCTGGTGGAGATCATATTCACTCTGG
Isopyrum	ACATTTCCGTGTACTAGCTAAAGCGTTACGTATGTCTGGTGGAGATCATATTCACTCTGG
Souliea	ACACTTCCGTGTACTAGCTAAAGCGTTACGTATGTCTGGTGGAGATCATATTCACTCTGG
Thalictrum	ACATTTCCGTGTACTAGCTAAAGCGTTACGTATGTCTGGTGGAGATCATATTCACTCCGG
Trollius	GCATTTTCGTGTACTAGCTAAAGCGTTACGTATGTCTGGTGGAGATCATATTCACGCTGG
Xanthorhiza	ACATTTCCGTGTACTAGCTAAAGCGTTACGTATGTCTGGTGGAGATCATATTCACTCTGG
Hydrastis	GCATTTCCGTGTACTAGCTAAAGCGTTACGTATGTCTGGCGGAGATCATATTCACGCTGC
Caulophyllum	ACATTTCCGTGTACTAGCTAAAGCGTTACGTATGTCTGGTGGAGATCATATTCACTCTGG
Cocculus	ACATTTCCGTGTACTAGCTAAAGCGTTACGTATGTCTGGTGGAGATCATATTCACTCTGG

Actaea	TACCGTAGTAGGTAAACTGGAAGGGGAAAGAGAGATCACCTTGGGCTTTGTTGATTTACT
Adonis	TACCGTAGTAGGTAAACTGGAAGGGGAAAGAGAGATCACCTTGGGTTTTGTTGATTTACT
Anemonopsis	TACCGTAGTAGGTAAACTGGAAGGGGAAAGAGAGATCACCTTGGGTTTTGTTGATTTACT
Beesia	TACCGTAGTAGGTAAACTGGAAGGGGAAAGAGAGATCACCTTGGGTTTTGTTGATTTACC
Caltha	CACCGTAGTAGGTAAACTGGAAGGGGAAAGAGAGATCACCTTGGGCTTTGTTGATTTACT
Cimicifuga	TACCGTAGTAGGTAAACTGGAAGGGGAAAGAGAGATCACCTTGGGCTTTGTTGATTTACT
Clematis	TACCGTAGTAGGTAAACTGGAAGGGGAAAGAGAGATCACCCTGGGCTTTGTTGATTTACT
Coptis	GACCGTAGTAGGTAAACTAGAAGGGGAAAGAGAGATCACCCTAGGCTTTGTTGATTTACT
Eranthis	TACCGTAGTAGGTAAACTGGAAGGGGAAAGAGAGATCACCTTGGGCTTTGTTGATTTACT
Isopyrum	TACCGTAGTAGGTAAACTAGAAGGGGAAAGAGAGATCACCTTGGGCTTTGTTGATTTACT
Souliea	TACCGTAGTAGGTAAACTGGAAGGGGAAAGAGAGATCACCTTGGGCTTTGTTGATTTACT
Thalictrum	TACCGTAGTAGGTAAACTAGAAGGGGAAAGAGAGATCACCTTGGGCTTTGTTGATTTACT
Trollius	TACCGTAGTAGGTAAACTGGAAGGGGAAAGAGAGATCACCTTGGGTTTTGTTGATTTACT
Xanthorhiza	GACCGTAGTAGGTAAACTAGAAGGGGAAAGAGAGATCACCCTGGGCTTTGTTGATTTACT
Hydrastis	TACCGTAGTAGGTAAACTAGAAGGAGAAAGAGAGATCACCTTGGGCTTTGTTGATTTACT
Caulophyllum	CACCGTAGTAGGTAAACTGGAAGGGGAAAGAGATATCACCCTGGGCTTTGTTGATTTATT
Cocculus	CACCGTAGTAGGTAAACTGGAAGGGGAAAGAGATATCACCCTGGGCTTTCTTGATTTATT

Appendix 1. continued.

Actaea	ACGTGATGATTTTATTGAAAAAGACCGAAGTCGCGGTATTTACTTCACTCAAGACTGGGT
Adonis	ACGTGATGATTATATTGCAAAAGA?CGAAGTCGCGGTATTTACTTCACCCAAGATTGGGT
Anemonopsis	ACGTGATGATTTTATTGAAAAAGACCGAAGTCGCGGTATTTACTTCACTCAAGACTGGGT
Beesia	ACGTGATGATTTTATTGAAAAAGACCGAAGTCGCGGTATTTACTTCACTCAAGACTGGGT
Caltha	ACGTGATGATTTCATTGAAAAAGACCGAAGTCGCGGTATTTACTTCACTCAAGATTGGGT
Cimicifuga	ACGTGATGATTTTATTGAAAAAGACCGAAGTCGCGGTATTTACTTCACTCAAGACTGGGT
Clematis	ACGTGATGATTTTATTGAAAAAGACCGAAGTCGCGGTATTTACTTCACTCAAGATTGGGT
Coptis	ACGCGACGATTTTATTGAAAAAGACCGAAGTCGCGGTATTTACTTCACTCAAGATTGGGT
Eranthis	ACGTGATGATTTTATTGAAAAAAACCGAAGTCGCGGTATTTACCTCACTCAAGACTGGGT
Isopyrum	ACGTGATGATTTTATTGAAAAAGACCGAAGTCGCGGTATTTACTTCACTCAAGATTGGGT
Souliea	ACGTGATGATTTTATTGAAAAAGACCGAAGTCGCGGTATTTACTTCACTCAAGACTGGGT
Thalictrum	ACGTGATGATTTTATTGAAAAAGACCGAAGTCGCGGTATTTACTTCACTCAAGATTGGGT
Trollius	ACGTGATGATTATATTGAAAAAGACCGAAGTCGCGGTATTTACTTCACCCAAGATTGGGT
Xanthorhiza	ACGCGACGATTTTATTGAAAAAGACCGAAGTCGCGGTATTTACTTCACTCAAGATTGGGT
Hydrastis	ACGTGATGATTTTATTGAAAAAGACCGAAGTCGCGGTATTTATTTCACTCAAGATTGGGT
Caulophyllum	ACGTGATGATGTTATTGAAAAAGACCGAAGTCGCGGTATTTATTTCGCCCAAGATTGGGT
Cocculus	ACGTGATGATGTTATTGAAAAAGACCGAAGTCGCGGTATTTATTTCGCCCAAGATTGGGT
Actaea	CTCTCTACCAGGCGTTCTGCCTGTTGCTTCAGGGGGTATTCACGTTTGGCATATGCCCGC
Adonis	TTCTCTGCCAGGTGTTCTGCCCGTTGCTTCAGGGGGTATTCACGTTTGGCATATGCCTGC
Anemonopsis	CTCTCTACCAGGCGTTCTGCCCGTTGCTTCAGGGGGTATTCACGTTTGGCATATGCCCGC
Beesia	CTCTCTACCAGGCGTTCTGCCCGTTGCTTCAGGGGGTATTCACGTTTGGCATATGCCCGC
Caltha	CTCTCTACCAGGTGTTCTACCCGTTGCTTCAGGGGGTATTCACGTTTGGCATATGCCTGC
Cimicifuga	CTCTCTGCCAGGCGTTCTGCCTGTTGCTTCAGGGGGTATTCACGTTTGGCATATGCCCGC
Clematis	CTCTCTACCAGGCGTTTTGCCCGTTGCTTCGGGGGGTATTCACGTTTGGCATATGCCTGC
Coptis	ATCTCTACCAGGTGTTCTGCCTGTTGCTTCAGGGGGTATTCACGTTTGGCATATGCCTGC
Eranthis	CTCTCTACCAGGCGTTCTGCCTGTTGCTTCAGGGGGTATTCACGTTTGGCATATGCCCGC
Isopyrum	CTCTCTACCAGGTGTTCTGCCCGTTGCTTCGGGGGGTATTCACGTTTGGCATATGCCTGC
Souliea	CTCTCTACCAGGCGTTCTGCCTGTTGCTTCAGGGGGTATCCACGTTTGGCATATGCCCGC
Thalictrum	CTCTCTACCAGGTGTTCTGCCTGTTGCTTCAGGGGGTATTCACGTTTGGCATATGCCTGC
Trollius	CTCTCTGCCAGGTGTTCTGCCCGTTGCTTCAGGGGGTATTCACGTTTGGCATATGCCTGC
Xanthorhiza	ATCTCTACCAGGTGTTCTGCCTGTTGCTTCAGGGGGTATTCACGTTTGGCATATGCCTGC
Hydrastis	CTCTCTACCAGGTGTTTTGCCCGTTGCTTCAGGGGGTATTCACGTTTGGCATATGCCTGC
Caulophyllum	CTCTCTACCAGGGGTTCTGCCCGTGGCTTCAGGGGGTATTCATGTTTGGCATATGCCTGC
Cocculus	CTCTCTACCAGGGGTTCTGCCCGTGGCTTCAGGGGGTATTCATGTTTGGCATATGCCTGC

Appendix 1. continued.

Actaea	TCTGACCGAGATCTTTGGGGATGATTCCGTACTACAGTTCGGTGGAGGAACTTTAGGACA
Adonis	TCTGACCGAGATCTTTGGGGATGATTCCGTACTACAGTTCGGTGGAGGAACTTTAGGACA
Anemonopsis	TCTGACCGAGATCTTTGGAGATGATTCCGTACTACAGTTCGGTGGAGGAACTTTAGGACA
Beesia	TCTGATCGAGATCTTTGGAGATGATTCCGTACTACAGTTCGGTGGAGGAACTTTAGGACA
Caltha	TCTGACCGAGATCTTTGGGGATGATTCCGTACTACAGTTTGGTGGAGGAACCTTAGGGCA
Cimicifuga	TCTGACCAGAATCTTTGGGGATGATTCCGTACTACAGTTCGGTGGAGGAACTTTAGGACA
Clematis	TCTAACCGAGATCTTTGGGGATGATTCCGTACTACAGTTCGGCGGAGGAACTTTAGGACA
Coptis	TTTGACCGAGATCTTTGGGGATGATTCCGTACTACAGTTTGGTGGAGGAACTTTAGGACA
Eranthis	TCTGACCGAGATCTTTGGGGATGATTCCGTACTACAGTTCGGTGGAGGAACTTTAGGACA
Isopyrum	TCTAACCGAAATCTTTGGGGATGATTCCGT?CTACAGTTCGGTGGAGGAACTTTAGGACA
Souliea	TCTGACCGAGATCTTTGGGGATGATTCCGTACTACAGTTCGGTGGAGGAACTTTAGGACA
Thalictrum	TCTAACCGAGATCTTTGGGGATGATTCCGTACTACAGTTCGGTGGCGGAACTTTAGGAAC
Trollius	TCTGACCGAGATCTTTGGGGATGATTCCGTACTACAGTTCGGTGGAGGAACTTTAGGACA
Xanthorhiza	T?T?ACCGAGATCTTTGGGGATGATTCCGTACTACAGTTCGGTGG?GGAACTTTAGGACA
Hydrastis	TCTGACCGAGATCATTGGGGATGATTCCGTACTACAGTTTGGTGGCGGAACTTTAGGACA
Caulophyllum	TCTGACCGAGATCTTTGGGGATGATTCCGTACTACAGTTCGGTGGAGGAACTTTAGGACA
Cocculus	TCTGACCGAGATCTTTGGGGATGATTCCGTACTACAGTTCGGTGGAGGAACTTTAGGACA

Actaea	CCCTTGGGGAAATGCACCGGGTGCCGTAGCTAATCGAGTAGCCCTAGAAGCCTGTGTACA
Adonis	CCCTTGGGGAAATGCACCGGGTGCCGTAGCTAATCGAGTAGCCCTAGAAGCCTGCGTACA
Anemonopsis	CCCTTGGGGAAATGCACCGGGTGCCGTAGCTAATCGAGTAGCCCTAGAAGCTTGTGTACA
Beesia	CCCTTGGGGAAATGCACCGGGTGCCGTAGCTAATCGAGTAGCCCTAGAAGCTTGTGTACA
Caltha	CCCTTGGGGAAATGCACCGGGTGCCGTAGCTAATCGAGTAGCCCTAGAAGCCTGTGTACA
Cimicifuga	CCCTTGGGGAAATGCACCGGGTGCCGTAGCTAATCGAGTAGCCCTAGAAGCCTGTGTACA
Clematis	CCCTTGGGGAAATGCACCAGGTGCCGTAGCTAATCGAGTCGCCCTAGAAGCCTGTGTACA
Coptis	CCCTTGGGGAAATGCACCGGGTGCCGTAGCTAATCGAGTAGCCCTAGAAGCCTGTGTACA
Eranthis	CCCTTGCGGAAATGCACCGGGTGCCGTAGCTAATCGAGTAGCCCTAGAAGCCTGTGTACA
Isopyrum	CCCTTGGGGAAATGCGCCGGGTGCCGTAGCTAATCGAGTAGCCCTAGAAGCCTGTGT?CA
Souliea	CCCTTGGGGAAATGCACCGGGTGCTGTAGCTAATCGAGTAGCCCTAGAAGCCTGTGTACA
Thalictrum	ACCTTGGGGAAATGCGCCGGGTGCCGTAGCTAATCGAGTAGCCCTAGAAGCTTGTGTACA
Trollius	CCCTTGGGGAAATGCACCGGGTGCCGTAGCTAATCGAGTAGCCCTAGAAGCCTGCGTACA
Xanthorhiza	CCCTTGGGGAAATGC?CCGGGTGCCGTAGCTAATCGAGTAGCCCTAGAAGCTTGTGTACA
Hydrastis	CCCTTGGGGAAATGCACCGGGTGCCGTAGCTAATCGAGTAGCCCTGGAAGCCTGTGTACA
Caulophyllum	CCCTTGGGGAAATGCACCGGGTGCCGTAGCTAATCGAGTAGCTCTAGAAGCGTGTGTACA
Cocculus	CCCTTGGGGAAATGCACCGGGTGCCGTAGCTAATCGAGTAGCTCTAGAAGCGTGTGTACA

Appendix 1. continued.

Actaea	AGCTCGTAATGAGGGCGTGATCTTGCTCGTGAAGGTAATGAAATTATCCGGAGGCTTG
Adonis	AGCTCGTAATGAGGGACGTGATCTTGCTCGTGAAGGTAATGAAATTATCCGTGAGGCTTG
Anemonopsis	AGCTCGTAATGAGGGACGTGATCTTGCTCGTGAAGGTAATGAAATTATCCGTGAGGCTTG
Beesia	AGCTCGTAATGAGGGACGTGATCTTGCTCGTGAAGGTAATGAAATTATCCGTGAGGCTTG
Caltha	AGCTCGTAATGAGGGACGTGATCTTGCTCGTGAAGGTAATGAAATTATCCGTGAGGCTTG
Cimicifuga	AGCCCGTAATGAGGGGCGTGATCTTGCTCGTGAAGGTAATGAAATTATCCGTGAGGCTTG
Clematis	AGCTCGTAATGAGGGACGTGATCTTGCTCGTGAAGGTAATGAAATTATCCGTGAGGCTTG
Coptis	AGCTCGTAATGAGGGACGTGATCTTGCTCGTGAAGGTAATGAAATTATCCGCGAGGCTTG
Eranthis	AGCTCGTAATGAGGGGCGTGATCTTGCTCGTGAAGGTAATGAAATTATTCGTGAGGCTTG
Isopyrum	AGCTCGTAATGAAGGACGCGATCTTGCTCGTGAAGGTAATGAAATTATCCGCGAGGCTTG
Souliea	AGCTCGTAATGAGGGGCGTGATCTTGCTCGTGAAGGTAATGAAATTATCCGTCAGCCTTG
Thalictrum	AGCTCGTAATGAAGGACGCGATCTTGCTCGTGAAGGTAATGAAATTATCCGTGAGGCTTG
Trollius	AGCTCGTAATGAGGGACGTGATCTTGCTCGTGAAGGTAATGAAATTATCCGTGA?GCTTG
Xanthorhiza	AGCTCGTAATGAAGGACGCGATCTTGCTCGTGAAGGTAATGAAATTATCCGTGAGGCTGC
Hydrastis	AGCTCGTAATGAGGGACGTGATCTTGCTCGTGAAGGTAATGAAATTATTCGTGAGGCTTG
Caulophyllum	AGCTCGTAATGAGGGACGTGATCTTGCTCGTGAAGGTAATGAAATTATCCGCGAGGCTTG
Cocculus	AGCTCGTAATGAGGGACGTGATCTTGCTCGTGAAGGTAATGAAATTATCCGCGAGGCTTG

Actaea	CAAATGGAGCGTT
Adonis	CAAATGGAGCCTT
Anemonopsis	CAAATGGAGCCTT
Beesia	CAAATGGAGCCTT
Caltha	CAAATGGAGTCTT
Cimicifuga	CAAATGGAGCCTT
Clematis	CAAATGGAGCCTT
Coptis	CAAATGGAGCCTT
Eranthis	CAAATGGAGCCTT
Isopyrum	CAAATGGAGCCTT
Souliea	CAAATGGAGCCTT
Thalictrum	CAAATGGAGCCTT
Trollius	CAAATGGAGCCTT
Xanthorhiza	CAAATGGAGCCTT
Hydrastis	CAAATGGAGCCCT
Caulophyllum	CAAATGGAGCCCT
Cocculus	CAAATGGAGCCCT

Appendix 2. 444 coded amino acids from rbcL sequences.

Actaea	S	V	G	F	K	A	G	V	K	D	Y	K	L	T	Y	Y	T	P	E	Y	A	P	K	D	T	D	T	L	A	A
Adonis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	.
Anemonopsis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
Beesia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Caltha	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
Cimicifuga	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Clematis	G	.	.	.	.	.	.	.	.	E	.	.	.	N	.	.	.	.	.	.	E	T	.	.	.	.	.	.	.	.
Coptis	S	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	T	P	.	.	.	.	.	.	.	.
Eranthis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Isopyrum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
Souliea	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
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Xanthorhiza	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
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Caulopyllum	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	D	.	V	T	.	.	.	.	I	.	.	.

Appendix 2. continued.

	F	R	V	T	P	Q	P	G	V	P	P	E	E	A	G	A	A	V	A	A	E	S	S	T	G	T	W	T	T	V
Actaea																														
Adonis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Cimicifuga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Xanthorhiza	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Caulophyllum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Appendix 2. continued.

	W	T	D	G	L	T	S	L	D	R	Y	K	G	R	C	Y	H	I	E	P	V	A	G	E	E	N	Q	Y	I	C
Actaea																														
Adonis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Cimicifuga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Caulophyllum	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.

Appendix 2. continued.

Actaea	Y	V	A	Y	P	L	D	L	F	E	E	G	S	V	T	N	M	F	T	S	I	V	G	N	V	F	G	F	K	?
Adonis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?	.	.	.	.	.	.
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Cimicifuga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?
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Xanthorhiza	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	A
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Caulophyllum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P

Appendix 2. continued.

Actaea	?	?	A	?	R	L	E	D	L	R	I	P	V	A	Y	V	K	T	F	Q	G	P	P	H	G	I	Q	V	E	R
Adonis	.	.	?	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Cimicifuga	?	?	?	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Clematis	L	R	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Coptis	?	?	?	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Xanthorhiza	Y	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Caulophyllum	.	R	A	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.

Appendix 2. continued.

	D	K	L	N	K	Y	G	R	P	L	L	G	C	T	I	K	P	K	L	G	L	S	A	K	N	Y	G	R	A	V
Actaea																														
Adonis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?	?	.	.	.	.	.	.	.	.	.
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Cimicifuga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
Clematis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Xanthorhiza	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hydrastis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Caulophyllum	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Appendix 2. continued.

Actaea	Y	E	C	L	R	G	G	L	D	F	T	K	D	D	E	N	V	N	S	Q	P	F	M	R	W	R	D	R	F	L
Adonis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Cimicifuga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Trollius	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Xanthorhiza	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Appendix 2. continued.

Actaea	F	C	A	E	A	I	Y	K	A	Q	A	E	T	G	E	I	K	G	H	Y	L	N	A	T	A	G	T	C	E	E
Adonis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?	.	.	.	.
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Cimicifuga	.	C	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
Clematis	.	.	.	.	.	I	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Coptis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Caulophyllum	.	.	.	.	.	L	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Appendix 2. continued.

	M	M	K	R	A	V	F	A	R	E	L	G	V	P	I	V	M	H	D	Y	L	T	G	G	F	T	A	N	T	S
Actaea	.	I	.	.	.	.	.	.	.	.	.	.	.	?	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Cimicifuga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Clematis	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Isopyrum	.	M	.	R	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Souliea	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Thalictrum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Trollius	.	?	.	.	.	.	.	.	.	.	.	.	.	?	I	.	.	.	.	.	.	?	.	.	.	.	.	.	?	.
Xanthorhiza	.	M	.	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.
Hydrastis	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	I	.	.	.	.	.	.	.	.	.
Caulophyllum	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	I	.	.	.	.	.	.	.	.	.

Appendix 2. continued.

Actaea	L	A	H	Y	C	R	D	N	G	L	L	L	H	I	H	R	A	M	H	A	V	I	D	R	Q	K	N	H	G	I
Adonis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M
Anemonopsis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
Beesia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Cimicifuga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
Clematis	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M
Coptis	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
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Isopyrum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Souliea	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
Thalictrum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.
Trollius	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M
Xanthorhiza	.	S	.	.	.	.	.	.	?	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
Hydrastis	.	A	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M
Caulophyllum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I

Appendix 2. continued.

	H	F	R	V	L	A	K	A	L	R	M	S	G	G	D	H	I	H	A	G	T	V	V	G	K	L	E	G	E	R
Actaea	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Beesia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Caltha	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cimicifuga	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
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Eranthis	.	.	R	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Isopyrum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Souliea	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Thalictrum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
Trollius	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.
Xanthorhiza	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
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Appendix 2. continued.

	E	I	T	L	G	F	V	D	L	L	R	D	D	F	I	E	K	D	R	S	R	G	I	Y	F	T	Q	D	W	V
Actaea	E	I	T	L	G	F	V	D	L	L	R	D	D	F	I	E	K	D	R	S	R	G	I	Y	F	T	Q	D	W	V
Adonis	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	?	.	.	.	.	.	.	.	.	.	.	.	.
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Cimicifuga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Xanthorhiza	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hydrastis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Caulophyllum	D	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.

Appendix 2. continued.

Actaea	S	L	P	G	V	L	P	V	A	S	G	G	I	H	V	W	H	M	P	A	L	T	E	I	F	G	D	D	S	V
Adonis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Anemonopsis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Beesia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Caltha	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cimicifuga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Clematis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Coptis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Isopyrum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?
Souliea	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V
Thalictrum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Trollius	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Xanthorhiza	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?	.	.	.	.	.	.	.	.	.
Hydrastis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.
Caulophyllum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Appendix 2. continued.

	L	Q	F	G	G	G	T	L	G	H	P	W	G	N	A	P	G	A	V	A	N	R	V	A	L	E	A	C	V	Q
Actaea																														
Adonis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Anemonopsis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Beesia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Caltha	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cimicifuga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Clematis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Coptis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Isopyrum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Souliea	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Thalictrum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Trollius	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Xanthorhiza	.	.	.	.	?	.	.	.	.	.	.	.	.	.	?	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hydrastis	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Caulophyllum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Appendix 2. continued.

Actaea	A	R	N	E	G	R	D	L	A	R	E	G	N	E	I	I	R	E	A	C	K	W	S	V
Adonis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L
Anemonopsis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Beesia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Caltha	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cimicifuga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Clematis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Coptis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Eranthis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Isopyrum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Souliea	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Thalictrum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Trollius	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?	.	.	.	.	.	.
Xanthorhiza	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	A	.	.	.	.
Hydrastis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	P
Caulophyllum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Appendix 3. Isolation of chloroplast DNA by sucrose step gradient centrifugation (Palmer, 1986).

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1) Prior to extraction, plants are kept in the dark for 3 to 5 days to reduce chloroplast starch levels.

2) Remove all the hard veins and cut into small pieces 2 to 5 cm^2 in surface area.

3) One hundred g of cut leaves is placed in 500 ml of ice-cold isolation buffer containing 0.35 M sorbitol, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1% BSA, and 0.1% 2-mercaptoethanol (v/v).

4) The leaf-buffer mixture is homogenized in a prechilled Waring blender for 3 to 5 5-sec. bursts at high speed.

5) The homogenate is filtered through four layers of cheesecloth (with squeezing) and then one layer of miracloth (without squeezing) and centrifuged at 1000 g for 15 min at 4^{o}.

6) The pellet is resuspended in 8 ml wash buffer (0.35 M sorbitol, 50 mM Tris-HCl, pH 8.0, 25 mM EDTA) by brushing, using a soft paint brush, and vigorous swirling of the centrifuge bottle.

7) The resuspended pellet is loaded onto a step gradient consisting of 10 ml of 60% sucrose (with 50 mM Tris-HCl, pH 8.0, 25 mM EDTA), overlayed with 10 ml of 45% sucrose (with 50 mM Tris-HCl, pH 8.0, 25 mM EDTA), and 10 ml of 15% sucrose (with 50 mM Tris-HCl, pH 8.0, 25 mM EDTA).

8) The step gradients are placed in a rotor and centrifuged at 25,000 rpm for 60 min at 4^{o}.

Appendix 3. continued.

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9) The chloroplast band at the 45-60% interface is removed with a wide bore pipette, diluted with 3-5 volumes wash buffer, and chloroplasts are pelleted by centrifugation at 3500 g for 20 min at 4° repeating this washing step for two times.

10) The chloroplast pellet is resuspended in wash buffer to a final volume of either 2 or 15 ml. One-tenth volume of a 10 mg/ml solution of self digested Pronase is then mixed in (1 hr at 37°).

11) Technical grade CsCl and ethidium bromide to a final concentration of 100 µg/ml and centrifuged in a rotor for 12-16 hr at 55,000 rpm.

12) Ethidium bromide is removed by three extractions with isopropanol saturated with NaCl and H_2O. Following the third extraction, the mixture is centrifuged briefly to enhance phase separation and to concentrate residual particulate matter at the isopropanol-aqueous interface.

13) The DNA-containing CsCl phase is then removed from below the isopropanol phase and add 2.5 volumes of ice-cold 100% ethanol and keep in -20° for overnight.

14) The chloroplast DNA is washed by 80% ethanol twice and after air dry, resuspended in TE (pH 8.0).

Appendix 4. ECL direct nucleic acid labelling and detection systems.

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1) Gel electrophoresis.
    1.1) Make a 1% agarose solution in 1x TAE buffer,.and add ethidium bromide, mix. pour into gel former, and insert gel comb to produce wells.
    1.2) Apply DNA samples to wells in gel.
    1.3) Apply current to gel and allow to run until bromophenol blue indicates that sample has run for a sufficient distance.
2) Processing the gel.
    2.1) Slide gel from gel-former into a suitable plastic box.
    2.2) Cover with depurination solution and begin agitation using an orbital shaker. Agitation should be continued for 15 minutes or at least until the bromophenol blue dye has at least until completely yellow.
    2.3) Discard depurination solution and rinse gel with distilled water.
    2.4) Cover gel with denaturation solution and begin agitation. Agitate for 15 minutes after the bromophenol dye has returned to its blue color.
    2.5) Discard solution and rinse gel with distilled water.

Appendix 4. continued.

2.6) Cover gel with neutralization solution and begin agitation for the approximate time it took for the dye to change color during denaturation, plus a further 15 minutes.

3) Capillary blotting.

3.1) Part fill a glass dish with 20x SSC and make a supporting platform. Cover it with three sheets of filter paper saturated with 20x SSC.

3.2) Place the gel on the filter paper.

3.3) Cut a sheet of nylon membrane to the size of the gel.

3.4) Place the membrane on top of the gel, without trapping air bubbles.

3.5) Place three sheets of filter paper, cut to size and wetted with 20x SSC on top of the membrane, avoiding trapping air bubbles.

3.6) Place a 5-7 cm stack of absorbent paper towels on top of the filter paper.

3.7) Place a glass on top of the paper towels and apply a weight, leave at least 3-4 hours.

4) Processing the blot.

Alkali fixation: Place membrane, DNA side up, onto filter paper saturated with 0.4 M NaOH for 5 minutes followed by a gentle rinse in 5x SSC for 1 minute.

5) Preparation of labelled probe.

Appendix 4. continued.

5.1) Boil the double-stranded DNA for 10 minutes.

5.2) Immediately cool the DNA on ice for 5 minutes. Spin reaction tube briefly in a microcentifuge to settle the liquid in the bottom of the tube.

5.3) Add an equivalent volume of DNA labelling reagent to the cooled DNA, which will now be single-stranded.

5.4) Add the glutaraldehyde solution; use a volume equivalent to the volume of the labelling reagent.

5.5) Spin reaction tube briefly in a microcentrifuge to settle the reaction mixture in the bottom of the tube, and incubate for 10 minutes at 37°C.

6) Hybridization.

6.1) At room temperature, take the hybridization buffer and add NaCl to 0.5 M a concentration. Add 5% w/v blocking agent and quickly mix the blocking agent into the buffer. Mix by roller mixer for at 1 hour at 42°C.

6.2) Add the labelled DNA probe to the prehybridization buffer and mix gently. The incubation with agitation at 42°C overnight.

7) Washing the membrane.

7.1) Remove blot from hybridization medium, place in a clean container and cover with an excess of primary wash

Appendix 4. continued.

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buffer containing urea should be incubated with agitation for 20 minutes at 42°C.

7.2) Discard wash buffer and replace with the equivalent volume of fresh primary wash buffer, incubate with agitation for a further 20 minutes at 42°C.

7.3) Discard wash buffer. Place blot in fresh container and add an excess of secondary wash buffer. Incubate with agitation for 10 minutes at room temperature.

7.4) Discard wash buffer and replace with an equivalent volume of fresh secondary wash buffer. Incubate with agitation for a further 5 minutes at room temperature.

8) Signal generation/detection.

8.1) Take the detection reagents that are supplied and mix an equal volume of detection solution 1 with detection solution 2 to give sufficient to cover the blot.

8.2) Remove excess wash buffer by placing blots on paper towels for 1 minute. Transfer blot to fresh containers. Add the mixed detection reagents directly to the blots on the face carrying the DNA.

8.3) Incubate for precisely 1 minute at room temperature.

8.4) Drain off excess detection buffer and wrap blots in SaranWrap.

8.5) Place the blots, DNA side up, in the film cassette.

Appendix 4. continued.

8.6) Switch off the lights and place a sheet of autoradiography film on top of the blots, close the cassette and expose for 10-20 minutes.

8.7) Remove film and develop the film for interpret results.

Appendix 5. DNA Isolation by CTAB method (Doyle & Doyle, 1987a; Doyle & Dickson, 1987b).

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1. Samples were ground in a mortar and pestle with liquid nitrogen.
2. Add a spatula tip of fine N_2 powdered tissue into 1.5 ml eppendorf tubes which contain 500 µl of warm 2X CTAB Extraction Buffer (2X CTAB = 2% hexadecyltrimethylammonium bromide (Sigma), 100 mM Tris-HCl pH 8.0, 1.4M NaCl, 20 mM EDTA, 0.2% 2-mercaptoethanol) and place in 60°C water bath for 2 hours.
3. Extraction were followed by adding an equal volume of chloroform by gently iverting the tube for 10 minutes then centrifuge at 12,000 rpm for 5 minutes.
4. The upper aqueous phase was removed to a new eppendorf and extracted with 400 µl of chloroform-isoamyl alcohol (24:1).
5. Repeat the same step as before. The aqueous phase was removed to a new eppendorf tube, and the nucleic acids were precipitated by adding 300 µl of cold isopropanol.
6. The tube should be kept in -20°C for at least 2 hours.
7. Samples were spun at 12,000 rpm for 5 minutes and the pellet were suspended with 100 µl of TE, then washed by adding equal volume of phenol-chloroform (1:1).
8. Microcentrifuge 10 minutes, remove supernatant. Add 2.5 volume of ice-cold 100% ethanol to place at -20°C for overnight.

Appendix 5. continued.

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9. Microcentrifuge 10 minutes, remove the 100% ethanol and rinse the pellet by 80% ethanol twice and dry under vacuum. Resuspend in 50µl TE buffer.

Appendix 6. PCR cycle used.

Stage 1.	(one cycle)	
	94°C	2.0 minutes
	60°C	2.0 minutes
	72°C	3.0 minutes
Stage 2.	(35 cycles)	
	94°C	1.5 minutes
	60°C	2.0 minutes
	72°C	3.0 minutes
Stage 3.	(one cycle)	
	72°C	15.0 minutes
	15°C	9999

Appendix 7. Alkaline-SDS lysis Method.

1. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at 12,000g for 30 seconds at 4°C in a microfuge.

2. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

3. Resuspend the bacterial pellet in 100 µl of ice-cold Soulution I* by vigorous vortexing.

4. Add 200 µl of freshly prepared Soulution II.**

5. Add 150 µl of ice-cold Soulution III.***
   Close the tube and vortex it gently in an inverted position for 10 seconds to disperse Solution III through the viscous bacterial lysate.
   Store the tube on ice for 3 - 5 minutes.

6. Centrifuge at 12,000g for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh tube.

7. Add an equal volume of phenol:chloroform. Mix by vortexing. After centrifuge at 12,000g for 2 minutes at 4°C, transfer the supernatant to a fresh tube.

8. Precipitate the double-stranded DNA with 2 volumes of ethanol at room temperature. Mix by vortexing. Allow the mixture to stand for 5 minutes at room temperature.

9. Centrifuge at 12,000g for 5 minutes at 4°C in a microfuge.

10. Remove the supernatant by gentle aspiration.

11. Rinse the pellet of double-strand DNA with 1 ml of 70% ethanol at 4°C.

12. Redissolve the nucleic acids in 50 µl of TE (pH 8.0) and store the DNA at -20°C.

Appendix 7. Continued.

*:	Solution I:	50 mM glucose	
		25 mM Tris·Cl (pH 8.0)	
		10 mM EDTA (pH 8.0)	
**:	Solution II:	0.2 N NaOH	
		1% SDS	
***:	Solution III:	5 M potassium acetate	60.0 ml
		glacial acetic acid	11.5 ml
		H_2O	28.5 ml

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