Distribution and Molecular Phylogeny of *Artemisia* Plants from Gilgit-Baltistan, Pakistan

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2014-2019

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In the Name of Allah, the Most Compassionate, the Most Merciful

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FINAL APPROVAL

It is certified that we have read the thesis submitted by Mr. Adil Hussain and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for Ph.D. degree in Biotechnology.

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A thesis submitted to the Department of Biological Sciences,
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Dedicated to

My loving parents

Who introduced me to the joy of reading from birth,

Enabling such a study to take place today

DECLARATION

I hereby declare that the work present in the following doctoral thesis is my own effort, except where otherwise acknowledged and that the dissertation is my own composition.No part of the thesis has been previously presented for any other degree.

Date 26/02/2019

AdilHussain

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LIST OF ABBREVIATIONS

^0C	Degree Celsius	LM	Light Microscopy
µL	Micro liter	M	Marker
AA	Adenine base pair	M	Meter
AB	Abaxial	mm	millimeter
ABS	Absinthium	MAFFT	Multiple Alignment using Fast Fourier Transform
AD	Adaxial	MEGA	Molecular Evolutionary Genetics Analysis
AG	Adenine Guanine	$MgCl_2$	Magnesium chloride
ART	Artemisia	ML	Maximum Likelihood
BIC	Bayesian Information Criterion	MP	Maximum Parsimony
BLAST	Basic local alignment search tool	MPT's	Maximum Parsimony trees
bp	Base pair	MSA's	Multiple sequence alignments
cDNA	Complementary Deoxyribonucleic acid	MVSP	Multivariate Statistical package
CC	Cytosine base pair	NCBI	National Center for Biotechnology Information
Cm	Centimeter	ng	Nano gram
cpDNA	Chloroplast Deoxyribonucleic acid	NJ	Nighbor Joining
CT	Cytosine Thymine	nrDNA	Nuclear ribosomal Deoxyribonucleic acid
CTAB	Cetyltrimethylammonium bromide	OUT	Out transforming unit
ddH_2O	Double distilled water	PCR	Polymerase Chain Reaction
DMSO	Dimethyl sulfoxide	P/E	Polar and Equatorial Ratio
DNA	Deoxyribonucleic acid	pH	Power of hydrogen ions
dNTPs	Di-nucleotidetphosphates	PHYLIP	PHYLogeny Inference Package
DRA	Dracunculus	PIC	Prior Inform Consent
EDTA	Ethylene di-Amine tetra acetic acid	PMNH	Pakistan Museum of Natural History
EMBL	European Molecular Biology Laboratory	PVPP	Polyvinylpolypyrrolidone
ETS	External Transcribed Spacer	rpm	Revolutions per minute
FACTOR	Multistate to binary recoding program	RNA	Ribonucleic acid
FISH	Florescent In situ Hybridization	SEM	Scaning Electron Microscopy
g	Grams	Spp.	Species
GARLI	Genetic Algorithm for Rapid Likelihood Inference	Sq.km	Square kilometre
GB	GenBank	Sub g.	Sub genus
GG	Guanine base pair	TAE	TRIS Aminomethane- EDTA
GIS	Global Imaging System	Taq	Thermophilus Aquaticus
GPS	Global Positioning System	TBE	TRIS-Borate-EDTA
GT	Guanine thymine	Temp	Temperature
ITS	Internal Transcribed Spacer	TT	Thymine base pair
IUCN	International Union for Conservation of Nature	UPGMA	Unweighted Pair Group Method with Arithmetic Mean
Km	Kilometer	UV	Ultraviolet

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1. **Adil Hussain**, Muhammad Qasim Hayat, Sumaira Sahreen, Syed Ali Imran Bokhari. 2019. Unveiling the Foliar Epidermal Anatomical Characteristics of Genus *Artemisia* (Asteraceae) from Northeast (Gilgit-Baltistan), Pakistan. International Journal of Agriculture and Biology. 21(3): 630–638. DOI: 10.17957/IJAB/15.0938.

2. **Adil Hussain**, Muhammad Qasim Hayat, Sumaira Sahreen, Qurrat Ul Ain, Syed Ali Imran Bokhari. 2017. Pharmacological promises of genus *Artemisia*: A review. Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences 54 (4): 265–287.

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ADIL HUSSAIN

ABSTRACT

In Pakistan, currently 38 species of the genus *Artemisia* (Asteraceae) have been identified so far. These *Artemisia* species were found in different phytogeographical regions including the Gilgit-Baltistan region of Pakistan. In this doctoral thesis, emphasis have been given to the ethnobotany, floral morphology, foliar epidermal anatomy (Epidermal cells/ stomata types and diversity in trichomes), pollen morphology, phytogeography and molecular phylogeny of *Artemisia* species from Gilgit-Baltistan region of Pakistan.

This study explored some rare species of the genus *Artemisia* and their folk medicinal uses from Gilgit-Baltistan region of Pakistan. The areas with deprived documentation of traditional information were covered and the native traditional medicinal awareness of the *Artemisia* species was acknowledged. For the first time in this investigation, 15 *Artemisia* species were explored and presented in the form of plates. These ethnobotanicaly important species were, *Artemisia absinthium* L., *Artemisia annua* L., *Artemisia austriaca* Jacq., *Artemisia biennis* Willd., *Artemisia campestris* L., *Artemisia chamaemelifolia* Vill., *Artemisia herba-alba* Asso., *Artemisia indica* Willd., *Artemisia maritima* L. Ex Hook. F., *Artemisia rutifolia* sub sp., *Artemisia rutifolia* var., *Artemisia scoparia* Waldst. & Kit., *Artemisia sieversiana* Ehrhl. Ex Willd., *Artemisia verlotiorum* Lamotte., and *Artemisia vulgaris* L. The native people of different regions of Gilgi-Baltistan uses different parts of these *Artemisia* species as food, ornaments, fuel and for medicinal purposes and were employed against more than 30 different kinds of ailments.

The morphology of this genus is very complex and difficult to address. This is because the same species shows different forms under certain ecological conditions. Therefore, it was a dire need to revise the morphology of *Artemisia* species from Gilgit-Baltistan region of Pakistan. In this study, 66 morphological characters of 20 *Artemisia* species were nominated for the cladistics analysis of genus *Artemisia*. The consequential cladogram divided genus *Artemisia* into five major clades. Subgenus *Artemisia* and subgenus *Absinthium* were dispersed between the clades of other sections and appeared as a polyphyletic. All the remaining sections were found to be monophyletic. Subgenus *Seriphidium* was grouped with *Artemisia* clade which authenticates its recombination with the genus *Artemisia*.

The micro-morphological attributes of foliar epidermal cells of 19 *Artemisia* species and stomata types of 17 *Artemisia* species using SEM and LM were evaluated. Primary

objective was to assess the diversity in epidermal cells of these important species for taxonomists to ensure their identification and delimitation. This study disclosed taxonomically essential epidermal attributes of some rare *Artemisia* species from Gilgit-Baltistan region of Pakistan. Epidermal cells investigated were varied from polygonal to irregular and elongate in shape, while wavy to smooth in margins. Investigation of stomata revealed five different types namely, anomocytic, diacytic, anomotetracytic, anisocytic, and paracytic, which were unequally distributed on both the abaxial and adaxial sides of studied *Artemisia* species.

The diversity in foliar trichomes of 17 *Artemisia* species obtained from Gilgit-Baltistan area of Pakistan were assessed and presented in the form of plates. A total of 10 main types of trichomes (Glandular and non-glandular) were noticed using light and scanning electron microscopic observations. The 4 glandular trichomes observed were peltate, pluricellular, capitate and thin necked trichomes, while the 6 non-glandular trichomes noticed were aduncate, unicellular calavate, conical type, stinging hair type, unicellular tector and unicellurar filiform.

The pollen morphology of 22 species of genus *Artemisia* from Gilgit-Baltistan region of Pakistan was assessed by means of scanning electron microscopy (SEM). This inquiry revealed pollen grains of *Artemisia* with tricolporate shape, characterized by globular symmetry (ellipsoid ball shaped from equatorial side and three lobed rounds from polar view) with few exceptions. Additionally, the pollens were marked with reduced spinules on their surfaces which are unique investigative character for the genus *Artemisia* of Asteraceae family. In this study, seven micromorphological characters (Pollen type, pollen shape, arrangement of spinules, exine sculpture, spinules base, equatorial width and polar length) of pollen grains of different *Artemisia* species were documented.

By using light microscopy and scanning electron microscopy, these 7 characters of *Artemisia* pollen were nominated for the cladistics and cluster analysis using PHYLIP and MVSP softwares. A data matrix was generated using these micro morphological traits of pollen for cladistics and cluster analysis.

The pollen traits are quite indicative for studies concerning the evolutionary kinships of species. The cladistic analysis based on the micromorphological characters of pollen confirmed the reunion of subgenus *Seriphidium* with *Artemisia*. In the subsequent cluster analysis, 5 groups within the genus *Artemisia* have been documented.

The investigations based on phytogeography of *Artemisia* as per collection data of this study confirmed the occurrence of majority of (44 %) *Artemisia* species in Gilgit district as compared to other districts of the region. This study substantiated that *A. maritima* and *A. sieversiana* are the most commonly found species in all parts of the studied districts. Moreover, *A. maritima* and *A. herba-alba* are most dominant in the hills and mountains of different districts of Gilgit-Baltistan region of Pakistan.

For the molecular phylogeny, the internal transcribed spacer (ITS), external transcribed spacer (ETS) of nuclear ribosomal DNA (nrDNA) and intergenic spacer (*psbA-trnH*) of choloroplast DNA (cpDNA) sequences of 28 *Artemisia* species collected from Gilgit-Baltistan region of Pakistan were utilized. The analyses have been done with maximum-likelihood (ML), maximum-parsimony (MP), neighbor joining (NJ) and Mr. Bayes algorithms. The results of this study confirmed the polyphyletic nature of subgenus *Artemisia* and *Absinthium*. Other subgenera like *Seriphidium, Tridentata, Pacifica* and *Dracunculus* were found to be monophyletic.

In this study, 10 undescribed taxa of *Artemisia* have also been observed. On the basis of phylogenetic results, these taxa were categorized in to different groups (Group I, II & III). The undescribed taxa with in group I was represented by one taxon (*A.* sp.-AD-H), whereas 4 undescribed taxa (*A.* sp.-A, *A.* sp.-B, *A.* sp.-C and *A.* sp.-E) were included in group II. Five undescribed taxa (*A.* sp.-D, *A.* sp.-F, *A.* sp.-G, *A.* sp.-H and *A.* sp.-I) were placed in group III. In all of them, 1 undescribed taxon within group I was placed in the subgenus *Dracunculus* with *Artemisia japonica* and *Artemisia desertorum*. Four undescribed taxa within group II were placed in the subgenus *Absinthium* with *Artemisia rutifolia* and the clade was designated as *A. rutifolia* complex. Five undescribed taxa within group III were also placed in the subgenus *Absinthium* with *Artemisia sieversiana*. Based on the current data and all available in literature, it is concluded that the morphological studies coupled with modern molecular techniques may lead to the clear infrageneric classification of the genus *Artemisia*. It will also clarify and characterize the undescribed taxa reported in this study.

CHAPTER 1

1. INTRODUCTION

Artemisia sp. L is a member of Asteraceae family, which is a polymorphic genus and is very significant from therapeutic and economic point of view. This genus is distributed mostly in the northern hemisphere's temperate sectors. A limited number of *Artemisia* species also occur in the southern hemisphere of the globe (Oberprieler *et al.*, 2009). This genus contains five hundred plants including both shrubs and herbs (Valles and McArthur, 2001) and designated as a prevalent and diverse genus of the Anthemideae community from Asteraceae (Martin *et al.*, 2003).

Maximum number of the *Artemisia* species, possess economic importance because they are utilized as fodder, therapeutics, feedstuff, aesthetics and soil binder, whilst a few taxa have been shown to be allergic and few are just noxious weeds. Many crops are damaged because they are vulnerable to their poisonous properties (Hayat *et al.*, 2009a). Few species of genus *Artemisia* are annual or biannual, while maximum numbers of species are perennial (Valles *et al.*, 2003).

It has been found that plants have the ability to generate greater amount of naturally occurring secondary metabolic substances, which may be significant pharmacologically. These essential metabolites may be phenolic glycosides, unsaturated lactones, saponins, flavonoids, phenols, glucosinolates and cyanogenic glycosides (Al-Zubairi *et al.*, 2009) and these constituents are exploited as a remedy for different ailments primarily, cancer, malaria, hepatitis, and microbial contagions. Additionally, some compounds from *Artemisia* are known to be potential insecticides and allopathic correspondingly.

The contemporary estimation by researchers showed that almost 87% of entire human ailments together with cancer, infections by bacteria and immunological syndromes are cured with naturally occurring products and their associated medicines and it is found that nearly 25% permitted drugs of the world are acquired from plant sources. Moreover, in the developing nations, approximately 80% of the population rely on traditional medicines from plants to fulfill their basic health care requirements. (Terra *et al.*, 2007)

Extracts obtained from *Artemisia* plants are used to cure many other ailments i.e. epilepsy, depression, psychoneurosis, insomnia, irritability, stress and anxiety (Walter *et al.*, 2003). The genus *Artemisia* also holds antiseptic, antispasmodic, antibacterial,

antitumor, antimalarial, hepato-protective and antirheumatic capabilities (Terra *et al.*, 2007). Among the deadly diseases, Malaria is considered to be a universal health problem leading to a death rate of around 1 million per year (Enserink, 2008). For the management of malaria and further kinds of sicknesses, Artemisinin is authentic and definite current drug of excellence. The compound Artemisinin is truly a sesquiterpene lactone produced by the glandular trichomes of *Artemisia annua* L. (Covello, 2008). This crucial compound comprises an endo-peroxide bridge with in the 1, 2, 3-trioxane structure (Figure 1.1) that holds noteworthy rank due to its antimalarial properties.

Artemisia annua is prevalent in China and have been utilized for nearly 2000 years with the aim of curing malaria (Hsu, 2006). Besides that, artemisinin is also documented in *Artemisia cina* (Aryanti *et al.*, 2001), *Artemisia apiacea* and *Artemisia lancea* (Hsu, 2006), and in the upper portion of *Artemisia sieberi* (Arab *et al.*, 2006). Moreover its occurrence is also confirmed in other species like *Artemisia absinthium* (Zia *et al.*, 2007), *Artemisia dubia* and *Artemisia indica* (Mannan *et al.*, 2008).

Presently, the supreme operational means to diminish the transmission rate of malaria is Artemisinin combination therapy (ACT) (Mutabingwa, 2005) and ACT is recommended by WHO as a prime cure for malaria instigated by *Plasmodium falciparum* (Li and Zhou, 2010). The malarial parasite's life cycle is presented in Figure 1.2. According to WHO, there were an estimated 219 million cases of malaria in 90 countries worldwide. The numbers of deaths worldwide from malaria has now dropped to about 435,000 in 2017 - in large part because of the use of artemisia-derived compounds coupled with ACT therapeutic approaches.

Artemisinin also showed its detrimental effects against some other parasites together with *Schistosoma* (Utzinger *et al.*, 2007), *Trypanomosa* (Nibret *et al.*, 2010), *Leishmania* (Sen *et al.*, 2007) and *Toxoplasma* (Dunay *et al.*, 2009). The antimalaric action of Artemisinin starts when the intra erythrocyte shapes of the parasite, hemoglobin is processed, and hematin is freed, and becomes harmful to the parasite. The parasite can diminish the harmful results of hematin when it is converted into hemozoin; nonetheless, chloroquine represses this reaction. Heme encourages artemisinin action that eventually destroys the parasite. The important reported compounds of *Artemisia* spp. around the world is given in Table 1.1.

Table 1.1. Pharmacologically important reported compounds in different *Artemisia* species.

Artemisia Spp.	Compounds	Reference
Artemisia absinthium L.	Myrcene, Linalool	Hänsel & Sticher, 2007
	Rutin, Luteolin, Quercetin, Myricetin, Apigenin, Spinacetin, p-hydroxyphenylacetic, p-coumaric, Chlorogenic, Vanillic	Craciunescu *et al.*, 2012
	5,6,3',5'-tetramethoxy 7,4'-hydroxyflavone, Artemitin, 5-hydroxy-3,3',4',6,7-pentamethoxyflavone,	Bora *et al.*, 2010
	Artabsin, Absinthin, Artemetin, Arabsin, Matricin, Isoabsinthin, Artemolin	Hänsel and Sticher, 2007
	Chamazulene	Kordali *et al.*, 2005
	Parishin B, Artenolide, 24X-ethylcholesta-7, Parishin C, 22-dien-3b-ol	Rastogi and Mehrotra, 2002
	α-bisabolol, matricin, β-curcumen, spathulenol	Wichtl, 2002
	Ergosterol, Stigmasterol, β – Sitosterol, Campesterol	Bora *et al.*, 2010
	Camphor	Nibret and Wink, 2010
	β-pinene	Rezaeinodehl and Khangholi,2008
	Trans-sabinyl acetate	López-Lutz *et al.*, 2008
	β-thujone	Rezaeinodehl and Khangholi, 2008
	β-thujone, Sabinene, Artemisia ketone, Trans-epoxyocimene, Trans-verbenol, Carvone, (E)-sabinyl acetate, Neryl 2-methylbutanoate, 1,8-cineole, Linalool and α-thujone, Curcumene, Neryl butyrate, Neryl 3-methylbutanoate, Chamazulene, Monoterpenes, Myrcene,	Orav *et al* , 2006
Artemisia afra Jacq. ex Willd.	Scopoletin, Acacetin, α-amyrin, Phytol, 12α,4α-dihydroxybishopsolicepolide, Pentacyclic triterpenoid betulinic acid	More *et al.*, 2012
	Camphor	Nibret and Wink, 2010
Artemisia annua L.	Artemisinin	Tu , 1981, Mannan *et al.*, 2010
	Artemisinin, Dihydro epideoxyarteanniun B, Deoxyartemisinin	Foglio *et al.*, 2002
*Artemisia biennis*Willd.	Camphor	Nibret and Wink, 2010
	Linalool	Padalia *et al.*, 2011
	Artemisia ketone	Viuda-Martos *et al* , 2010
	Artemisia ketone, 1,8-cineole, Camphor, α-pinene	Mazandarani *et al.*, 2012
	Para-Cymene, (2E)-Hexenal, Santolina triene, Alpha-Pinene, Benzaldehyde, Sabinene, Limonene, Terpinen-4-ol, Alpha-Terpinene, (E)-beta-Farnesene, (Z)-en-yn-Dicycloether, trans-ocimene, Farnesene, Camphor, α-pinene, Artemisia ketone, (Z)-beta-Ocimene,	López-Lutz *et al.*, 2008

Table 1.1. Continued...

Artemisia californica Less.	Jaceosidin, Jaceidin, Chrysoplenol	Allison *et al.*, 2016
	Eucalyptol, Camphor	Fontaine *et al.*, 2013
	Artemisia ketone, 1,8-cineole, Isothujone, Camphor, α-thujone	Halligan, 1973
Artemisia campestris L.	Caryophyllene oxide, Germacrene D	Judzentiene *et al*, 2010
Artemisia dracunculus L.	Trans-anethole, Limonene, Trans-ocimene	Sayyah *et al.*, 2004
	Trans-ocimene	López-Lutz *et al.*, 2008
	Artemisinin	Mannan *et al.*, 2010
	Trans-ocimene, Methyl chavicol, Chavicol, Cis- and trans-ocimene, Limonene	Rustaiyan & Faridchehr, 2014
	α-pinene, Camphene, α-thujene, Myrecene, 1,8-cineole, (Z)-β-ocimene, (E)-β-ocimene, α, Terpenolene, Elemicin, α-copaene, Trans-Anethole, Myrecene	Tak *et al.*, 2014
Artemisia frigida Willd.	Camphor, 1,8-cineole	López-Lutz *et al.*, 2008
	1,8-cineole, Cis-ρ-menth-2-en-1-ol, Borneol, Camphor, Bicyclogermacrene, Lavandulol,	Liu *et al.*, 2014
	Borneol, α-pinene, Camphene, Camphor, Terpine-4-ol, Bornyl acetate, Germacrene D, 1,8-cineole	Korolyuk and Tkachev, 2010
Artemisia herba-alba Asso.	Chrysanthenyl propionate	Shah *et al.*, 2011
	α- thujone, β-thujone, Cis-sabinol, Piperitone, Camphor, Terpinen-4-ol, Fenchol, Verbenol, Chrysanthenone, Widdrene, 1-Butanol, 3-methyl-,16-Diepoxyhexadecane β-Guaiene, α-longipinene, α–bulnesene, Acetic acid, butyl ester, acetate, Ethyl linoleate, Linoleic acid, α-Bisabolol oxide, Bergamotol, Z-α-trans, 1,2–15, , trans-(Z)-α, bisabolene epoxide, Farnesene epoxide E, α-bergamotene,	Tilaoui *et al.*, 2015
Artemisia indica Willd.	α-Pinene, β-Pinene, β-Myrecene, 1,8-cineole, Linalool, Chrysanthenyl, Divanone	Haider *et al.*, 2014
Artemisia maritima L.	Chrysanthenyl propionate, Elixene, α-Pinene, α-Terpinyl acetate, Artemone, Santolina alcohol, β-Pinene, 2,3-Dehydro-1,8-cineole, α-Terpinene, Bornyl acetate, Aromadendrene, Cis-Davanone, Camphene,	Shah *et al.*, 2011
	Eucalyptol, Camphor, Borneol, Bornyl acetate, Germacrene-D	Sharma *et al.*, 2014
	Camphor and 1,8-cineole	Stappen *et al.*, 2014

Table 1.1. Continued...

Artemisia scoparia Waldst. & Kitam.	1,8-cineole	Cha *et al.*, 2005
	p-cymene, β-myrcene	Singh *et al.*, 2010
	Limonene	Singh *et al.*, 2008
	β-pinene	Sharopov and Setzer, 2011
	γ-terpinene	Joshi *et al.*, 2010
	α-thujone, β-thujone	Farzaneh *et al.*, 2006
Artemisia sieversiana Willd.	Eucaliptol	Liu *et al.*, 2010
	Myrcene, 1,8-Cineol, α-Thujone, β-Thujone, p-Cymene, Camphor, Linalool, b-Caryophyllene, cis-p-Mentha -2-en-1-ol	Suleimenov *et al.*, 2009
Artemisia stelleriana Besser.	1,8-Cineole, Germacrene D, Vulgarone B, Artedouglasia oxides, Artedouglasia oxide, Artedouglasia oxide B, ASartedouglasia oxide C, Artedouglasia oxide D	Padalia *et al.*, 2016
Artemisia tridentata Nutt.	Camphor, 1,8-cineol, Nerol, Neryl, Himachalenes, Longifolene, Caryophyllene, and Acetylenic spiroethers	Jasbi et al., 2010
	2-isopropenyl-5-methylhexa-trans-3,5-diene-1-ol, 2,2-Dimethyl-6-isopropenyl-2H-pyran, 2,3-dimethyl-6-isopropyl-4H-pyran,	Gunuwardena *et al.*, 2002
Artemisia verlotiorum Lamotte.	α-thujone and Camphor	Haider *et al.*, 2006
Artimisia vulgaris L.	Sabinene, 1,8-Cineole, cis-Thujone, b-Pinene, Caryophyllene, trans-Thujone, Cadinene, Chrysanthenyl acetate, epi-α-Muurolol, Salvial-4(14)-en-1-one, Germacrene D, Artemisia ketone,	Judžentienė and Buzelytė, 2006
	Trans-Caryophyllene, 1,8-Cineol, Trans-Salvene, ß –Cubebene, 5-Humulene	Bamoniri *et al.*, 2010

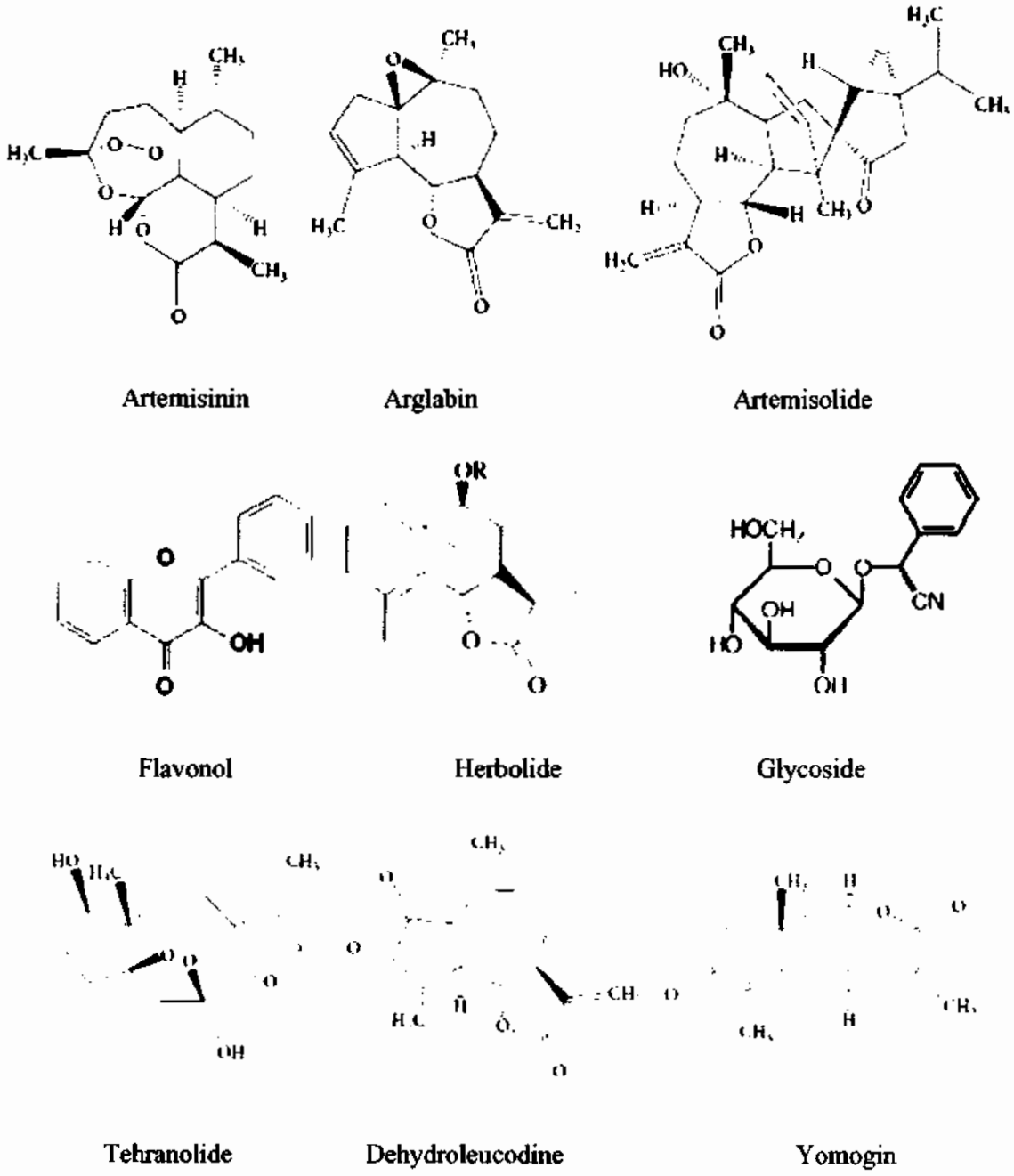

Figure 1.1. Structure of some bioactive compounds derived from different *Artemisia* species.
Source: Hussain *et al.*, (2017)

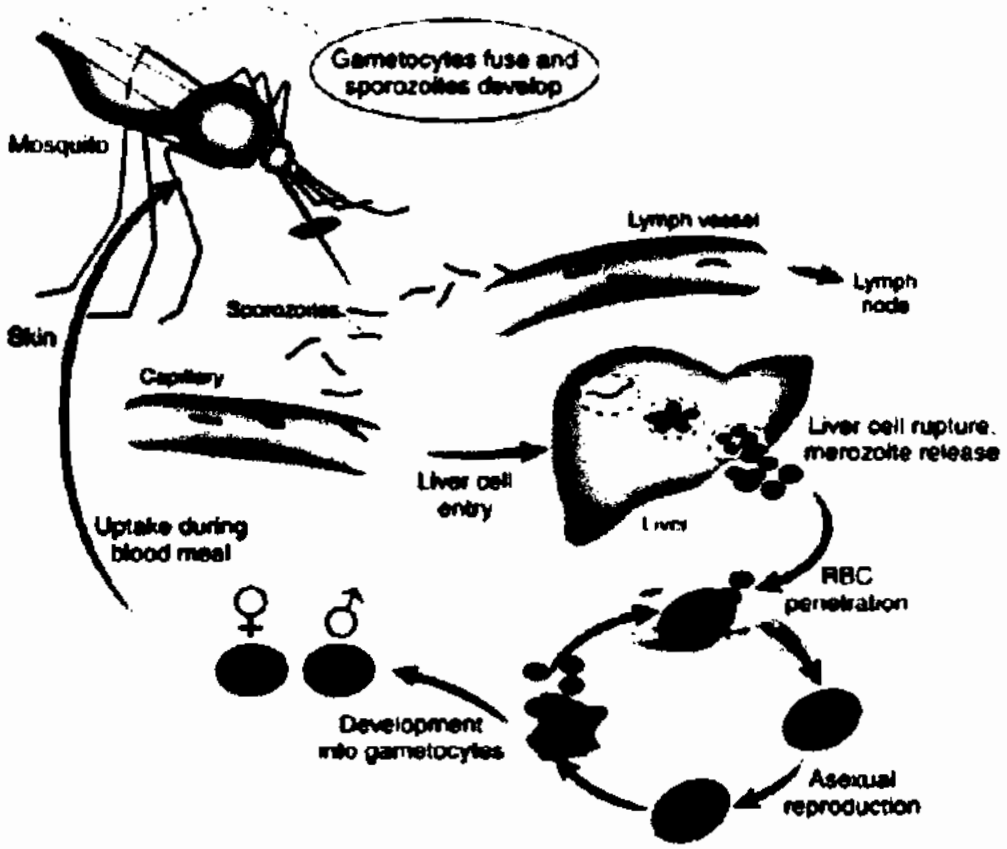

Figure 1.2. The life cycle of the malarial parasite (Adapted from Batista *et al.*, (2009).

Pakistan possesses a phenomenal climatic diversity and it ranges from the hot Sindh deserts to the comparatively cold forests of Khyber Pakhtunkhwa (KPK) formerly called the North West Frontier Province (NWFP). This fluctuating environment acts as an enriched place for a greater biodiversity (Nasir and Rafiq, 1995).

Currently, 38 to 42 different species of *Artemisia* have been botanically accredited and described from different areas of Pakistan including, Baluchistan, NWFP, North Punjab and the temperate areas of Gilgit-Baltistan and Kashmir (Ghafoor, 2002).

Wide-ranging drugs and constituents are obtained from different plants of the area and there is a great potential for the use of medicinally important plants (Ali 1995).

The ethno botanical studies in Pakistan have revealed that almost 400 to 600 plants among 5,700 species are found to be very crucial medicinally and they are only found in the high-altitude zones (Ali and Qaiser 1986).

According to IUCN (2015), 3,000 species of plants have been accredited from the region, where 124 are found important pharmacologically (IUCN, 1999).

It has been showed that the severe pressure on medicinal plants was posed by non-scientific and a saturated occurence of medicinal plants in several parts of the area. This leads to different problems in the recognition of how much medicinal plants are endangered. However, according to IUCN, 32,000 plant species are currently threatened which represents 13% of the estimated 250,000 plants.

Moreover, few species of *Artemisia* (Table 1.2) are specified as threatened and endangered in the IUCN red list because of a drastic decline in population. Their dispersal is harshly fragmented and there is unending decline in the populations due to collection, herbivores predation and less ability of adaptation in response to adverse climatic conditions.

Table 1.2. ***Artemisia*** **species in the red list of International Union for Conservation of Nature (IUCN).**

***Artemisia* Spp.**	**Status**	**Population trend**
Artemisia eriantha Var.	Least Concern	Stable
Artemisia genipi Weber.	Least Concern	Stable
Artemisia granatensis Boiss.	Endangered	Decreasing
Artemisia insipida Vill.	Critically Endangered	Unknown
Artemisia molinieri Quézel, M.Barbero & R.J.Loisel.	Vulnerable	Decreasing
Artemisia oelandica Besser.	Near Threatened	Decreasing
Artemisia pancicii Ronniger ex Dan & Mar.	Data Deficient	Decreasing
Artemisia umbelliformis Lam.	Least Concern	Stable
Artemisia campestris L.	Least Concern	Decreasing
Artemisia campestris ssp. bottnica	Near Threatened	Stable
Artemisia alba Turra.	Least Concern	Unknown
Artemisia absinthium L.	Least Concern	Stable
Artemisia laciniata Willd.	Data Deficient	Unknown
Artemisia santonicum L.	Least Concern	Decreasing
Artemisia vulgaris L.	Least Concern	Stable

Source: www.iucnredlist.org

Classification of the genus *Artemisia* is based primarily on its capitular morphology (Watson *et al.*, 2002). In the past, *Artemisia* genus was divided into three separate genera like *Absinthium*, *Artemisia* and *Abrotanum*. Linnaeus designated these three genera as a single genus. Moreover, Valles & McArthur (2001) documented four sections within *Artemisia* based on the fertility/sterility of disc florets or the appearance/nonappearance of ray florets: (1) *Artemisia* contains ray fertile pistillate florets; disc perfect fertile florets on glabrous receptacle, (2) *Absinthium* have pistillate ray fertile florets and disc perfect fertile florets on the hairy receptacle, (3) *Serphidium* is without ray florets, but have disc perfect fertile florets present on the glabrous receptacle, and (4) *Dracunculus* have pistillate ray fertile florets and disc staminate florets on the glabrous receptacle.

For the natural classification, *Artemisia* was divided into four distinct groups on the basis of floral characters employed as taxonomic marker. These include the subgenera *Artemisia* Tournefort, *Seriphidium* Besser, *Absinthium* (Tournefort) de Cand., and *Dracunculus* Besser (Martin *et al.*, 2003). Another new set of the genus named as *Tridantate* (Rydb.) was later proposed by McArthur *et al.*, (1981) which is widespread to North region of America.

Seriphidium was separated from the genus *Artemisia* by Ling (1982). Bremer & Humphries (1993) acknowledged the seperation done by Ling (1982). Nonetheless, molecular studies done by Kornkven *et al.*, (1998) and Watson *et al.*, (2002) again pooled *Artemisia* and *Seriphidium*. Their studies declined the seperation of subgenus *Artemisia* and *Seriphidium*. This raised a new debate about the classification of *Artemisia* on subgeneric level.

Studies of Hayat, (2011) again showed that *Seriphidium* and *Artemisia* are not distant groups. Malik *et al.*, (2017) showed subgenus *Serphidium* with two monophyletic clades.

Historical developments in the infrageneric classification of the genus *Artemisia* based on floral morphology are provided in Table 1.3.

Table. 1.3. Historical developments in the infrageneric classification of genus *Artemisia* based on floral morphology

Rank	Infrageneric Taxa					Reference
Genera	*Absinthium*	*Abortanum*	*Artemisia*			Tournefort, 1700
Genus	*Artemisia*					Linnaeus, 1735
Genera	*Artemisia*				*Oligosporus*	Cassini, 1817; Lessing, 1832
Sections	*Absinthium*	*Abortanum*	*Seriphidium*		*Dracunculus*	Besser, 1829
Sections	*Absinthium*	*Abortanum*	*Seriphidium*		*Dracunculus*	De Candolle, 1837
Subgenera	*Euartemisia*		*Seriphidium*		*Euartemisia*	Rouy, 1903
Subgenera	*Absinthium*	*Abortanum*	*Seriphidium*		*Dracunculus*	
Sections			*Seriphidium*	*Tridentatae*		Rydberg, 1961
Subgenera	*Artemisia*		*Seriphidium*		*Dracunculus*	Polyakov, 1961
Subgenera	*Absinthium*	*Artemisia*	*Seriphidium*		*Dracunculus*	Persson, 1974
Sections	*Artemisia*				*Dracunculus*	Tutin *et al.*, 1976
Subgenera	*Artemisia*		*Seriphidium*	*Tridentatae*	*Dracunculus*	McArthur *et al.*, 1981
Subgenera	*Artemisia*		*Seriphidium*		*Dracunculus*	Podlech, 1986
Genera	*Artemisia*				*Seriphidium*	
Subgenera	*Absinthium*		*Dracunculus*		*Seriphidium*	Ling, 1991
Genera	*Artemisia*			*Seriphidium*		Bremer and Humphries, 1993
Genera	*Artemisia*				*Seriphidium*	Ghafoor, 2002
Morphology	Heterogamous capitula having both pistillate ray florets and disc florets			Homogamous capitula having bisexual disk florets		Watson *et al.*, 2002; Rashmi *et al.*, 2016
	Bisexual disk florets		Staminate disk florets			
	Receptacle hairy	Receptacle glabrous		Cosmopolitan	Only endemic to North America	
Legends						

1.1. Introduction to the Study Area

Gilgit-Baltistan (Formerly called Northern areas) is a far flung region of Pakistan situated between 34.6°-37.4°N and 74°–77.5°E with the area of almost 45224 km^2. Altitude of this region ranged ±1400m to 8615m. The eastern side have borders with China connected by the Khunjerab pass with the altitude of ±4634 m. The north western border of this region is connected with Afghanistan and western and southern sides are delimited with the occupied Jamu and Kashmir.

Administratively, the area is separated into seven major districts i.e. Gilgit, Hunza Nagar, Skardu, Ghanche, Diamer, Astore and Ghizer (Figure 1.3) and further categorized into tehsils and sub-divisions. Population of the area is 546538 where 95,607 are urban and the rest of population (450931) is rural.

According to estimation, the annual population growth rate of this region is 2.47%. Gilgit-Baltistan is well-known for having world renowned mountain ranges like the Karakorum, Hindukush and the Hamalayas. There are a lot of peaks with the height of above 7000m including Godwin Austin (K-2, 8611m), Rakaposhi (7788m) and Deran peak (7268m). Baltoro is one of the biggest glaciers stretched for about 62 km with the area of 529 km^2 (Anonymous, 2003).

Due to the high mountainous topography and difference in altitude, a larger area of the region is uninhabitable.

These high mountain ranges have arctic climate (Khan, 1995) and also have impact on the climatic changes with mild summer and cool winter. Temperature may range from less than -10°C in winter and extremes of nearly 40°C in summer in the bottom valleys.

This area is well known for a great biodiversity of plants (Shinwari, 2010) and exports medicinal herbs, because of being the hub of important medicinal plants (Shinwari and Gilani 2003).

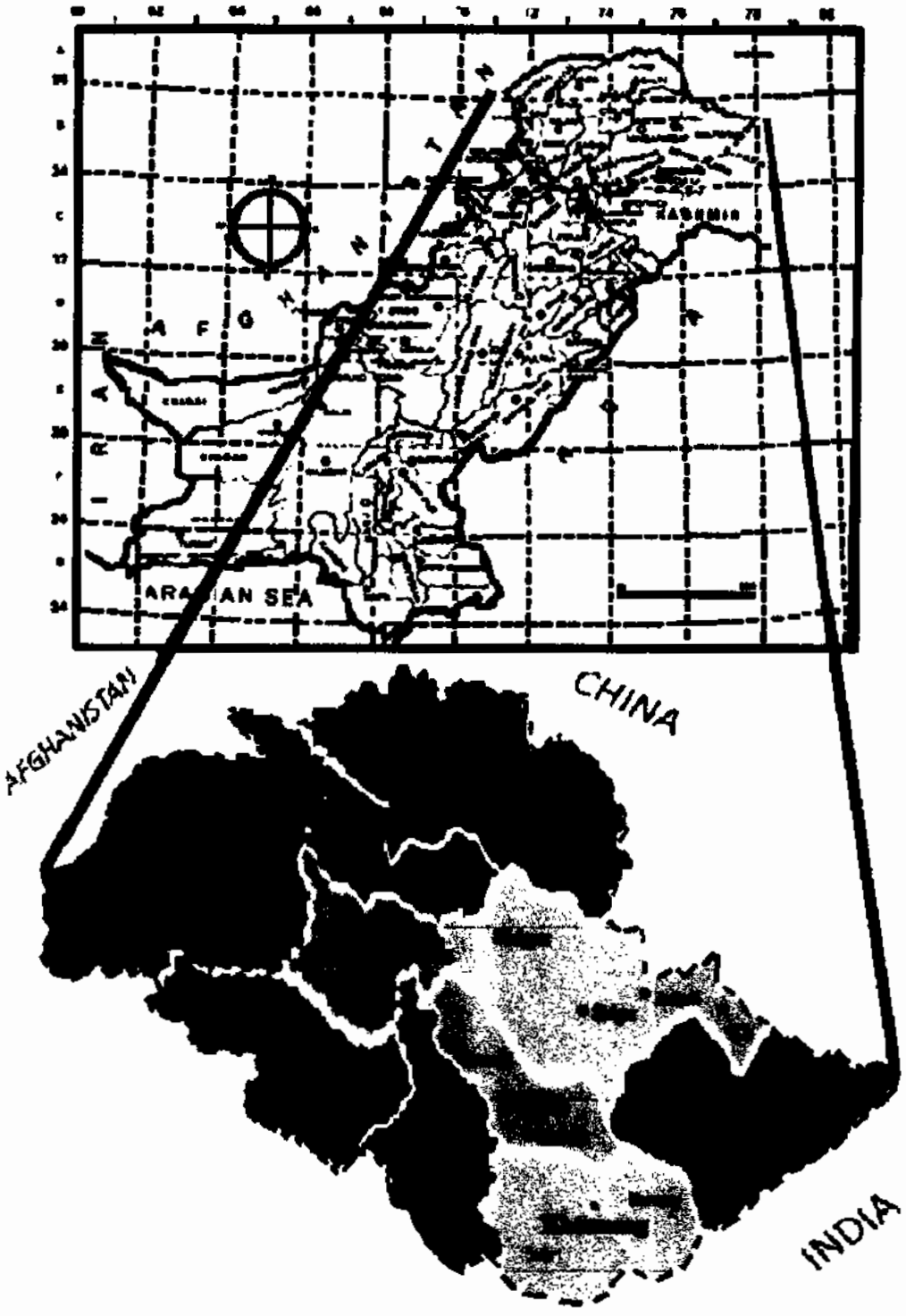

Figure 1.3. Map of Gilgit-Baltistan region of Pakistan showing the division of its districts.

1.2. Justification of Present Study

The taxonomy of genus *Artemisia* persisted controversial for many years. Due to the large population, there exist infra generic classification and species delimitation problems in this genus. In a lot of investigations, *Artemisia* and its allies were segregated as seperate genera while other studies pooled them again on the basis of different parameters.

The subgeneric level classifications based on morphology and the fertility of florets were also conducted.

After the joining and separations of *Artemisia* and its allies on the basis of their sectional or subgeneric rank, five to six groups were recognized within the genus. These groups include, *Artemisia* Tournefort, *Absinthium* (Mill.) DC *Seriphidium* (Besser) Besser, *Dracunculus* Besser, *Pacifica* and *Tridentatae* (Rydb.) McArthur.

In the recent molecular investigations, these grouping to some extent have been established a final classification of this genus, but its classification is still confusing and under investigation by many research groups.

Expectantly, this study may help in tailoring the subgeneric classification of the genus *Artemisia* by employing multiple taxonomic methods.

Many researchers (see Chapter 2, section 2.1) conducted ethnobotanical studies on the genus *Artemisia* in Pakistan, but the ethnobotanical perspectives of *Artemisia* was not specifically reported from Gilgit-Baltistan region. Therefore, the ethnobotany of *Artemisia* species from Gilgit-Baltistan region was done with the aim of developing a deeper understanding of its pharmacological importance.

Some researchers (see Chapter 2 section 2.2) have previously worked on the morphology of different *Artemisia* species from Pakistan, but their studies didn't focused towards the cladistic interactions and classification of some rare *Artemisia* species from Gilgit-Baltistan. Consequently in this study, a cladistics analysis based on morphological characteristic of *Artemisia* from Gilgit-Baltistan region of Pakistan was done.

There exists insufficient data regarding the foliar epidermal anatomy of *Artemisia* species (see Chapter 2, section 2.3) and the comparative account of foliar anatomy of few *Artemisia* species has been done so far in Pakistan. In this research work, the foliar epidermal anatomy (Epidermal cells, stomatal diversity and varation in foliar trichomes) of

some rare and undescribed *Artemisia* species have been presented from Gilgit-Baltistan region of Pakistan.

The morphology of pollen grains of some *Artemisia* species was accomplished formerly (see Chapter 2, section 2.4) around the world including Pakistan, but majority of the *Artemisia* species from Gilgit-Baltistan region of Pakistan were not included in those investigations. Therefore, the features of *Artemisia* pollen grain from Gilgit-Baltistan region of Pakistan were scrutinized and the micromorphological data of *Artemisia* pollen were used for cladistic and cluster analysis.

The phytogeography of *Artemisia* was formed in the perspective of Gilgit-Baltistan region of Pakistan (see Chapter 2, section 2.5) and presented the arrangements of diversity/distribution of *Artemisia* in this region.

For the first time, this study presented a molecular phylogenetic study of *Artemisia* from the Northeast (Gilgit-Baltistan) region of Pakistan based on nrDNA internal transcribed spacer (ITS), external transcribed spacer (ETS) and cpDNA intergenic spacer (*psbA-trnH*) regions (see Chapter 2, section 2.6). The phylogenetic study determined the relationships of Northeastern Pakistani *Artemisia* and tested whether or not *Seriphidium* was nested within *Artemisia*. This study also determined the phylogenetic settlements of some undescribed taxa collected during field visits from Gilgit-Baltistan region of Pakistan.

1.3. Aims and Objectives

The main objectives of the present study were:

1. To explore medicinally important species of the genus *Artemisia* and investigation of their several ethnobotanical and ethno taxonomical attributes from Gilgit-Baltistan region of Pakistan.
2. To assess the floral morphological characteristics of *Artemisia* species and construction of cladogram based on floral morphological characteristics.
3. To explain the leaf epidermal cells characteristics with stomatal distribution in different *Artemisia* species from Gilgit-Baltistan region of Pakistan.
4. To investigate the diversity of leaf foliar trichomes among different species of *Artemisia* from Gilgit-Baltistan region of Pakistan.
5. To examine pollen characteristics of *Artemisia* species and performing cladistics and cluster analysis based on pollen micromorphological characteristics.
6. To unveil the phytogeographical distribution of genus *Artemisia* from Gilgit-Baltistan region of Pakistan.
7. To study molecular phylogeny using internal and external transcribed spacer (ITS and ETS) sequences of nuclear ribosomal DNA (nrDNA) and *psbA-trnH* intergenic spacer sequences of chloroplast DNA (cpDNA) of different *Artemisia* species from Gilgit-Baltistan region of Pakistan. To determine the phylogenetic relationships of Northeastern Pakistani *Artemisia*; To test whether or not *Seriphidium* is nested within *Artemisia*; To determine the phylogenetic placements of undescribed taxa collected during field work for this study, which do not match the morphological descriptions of any previously described species of *Artemisia*.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Ethnobotany of *Artemisia*

Ethnobotanical experiences of many communities around the world are utilized for the treatment of wide range of diseases. A lot of divergent communities hold their own acquaintance about the utilization of medicinally important plants (Osawaru and Dania-Ogbe, 2010). Primarily, the ethnobotanical investigations focus on providing knowledge about the traditional utilization of plants and secondly, this knowledge is employed for additional scientific revelations (Parada *et al.*, 2009). Ethno botanical studies also confirm that the indigenous knowledge about the conservation of biological diversity and cultural information is rendered in a proper way (Ibrar *et al.*, 2007). It has been shown that above 5000 different plant species from angiosperms have been employed for medicinal purpose globally and these plants are used against different health problems directly or indirectly (Govaerts, 2001).

The species of *Artemisia* are essentially found in central Asian, European and North American regions. They are typically perennial herbs dominating the vast steppe communities of Asia. Asia has the greatest concentration of species, with 174 in the ex-USSR, 150 accessions for China, 35 species found in Iran and about 50 species have been reported for Japan. The genus *Artemisia* has a therapeutic history reaching back over two millennia and species of this genus are extensively utilized in traditional remedies to treat diseases like malaria, cancer, hepatitis, inflammation and bacterial, fungal and viral infections.

Many researchers have reported the folk medicinal utilization of different *Artemisia* species with number of studies reported and number of disease treated in Pakistan (Khan, *et al.*, 2003; Qureshi *et al.*, 2002; Iqbal *et al.*, 2004; Hayat *et al.*, 2009a; Sardar and Khan, 2009; Ashraf *et al.*, 2010; Nadeem *et al.*, 2013 and references there in) as shown in Table 2.1 and 2.2. Other researchers (Bonet *et al.*, 1999; Gruenwald, 2000; Kershaw, 2000; VanWyk and Wink, 2004; Volpato *et al.*, 2009; Amiri and Joharchi, 2013; Bekele *et al.*, 2015 and references there in) have also reported the folk medicinal uses of *Artemisia* species around the world as shown in Table 2.3.

Artemisia abrotanum "southernwood" preparations are employed in the traditional medicine for the treatment of multiple sicknesses including the upper airway infections. This perennial plant is presently utilized predominantly for cosmetic and culinary reasons (Gruenwald, 2000).

Some *Artemisia* species like *A. absinthium*, *A. annua* and *A. vulgaris* have been utilized since the ancient times in most Asian and European countries (Proksch, 1992). One study reported that the digestibility performance of animals could be increased when wormwood (*Artemisia* sp.) was given in place of rice straw in diet (Ko *et al.*, 2006).

Other species of *Artemisia* have also been documented for their antidiabetic properties and have been used in many countries for diabetes, high blood pressure and stomach ailments treatment (Subramoniam *et al.*, 1996).

Artemisia absinthium leaf powder is conventionally used in Pakistan for the treatment of gastric problems and intestinal worms. Its seed powder is taken orally against rheumatism and is applied directly on teeth to relieve pain (Hayat *et al.*, 2009a). Amiri and Joharchi, (2013) reported the traditional utilization of *A. absinthium* as an anthelmintic and appetizer.

Artemisia annua also called "sweet wormwood" or "qinghao" is traditionally employed in China to treat fever and chills (Hong *et al.*, 2015). This plant although is originally native to Asia and Europe, but it is also cultivated in Africa and employed as tea against malaria. A significant constituent of *A. annua* is Artemisinin and it is identified as a potential anti-malarial compound. At present, artemisinin derived compounds are also taken with efficacy against drug-resistant *Plasmodium* contagions (Tu, 1981; Wright, 2002).

In the North region of Pakistan, decoction of *A. Annua* whole plant is used for the treatment of Malari. Its leaves are used to cure fever, common cold, cough and dried powder of leaves is taken against diarrhea (Hayat *et al.*, 2009a).

Artemisia afra, a native South African medicinal herb and is usually called as "Wilde-als" is extensively utilized against various health related problems like asthma, diabetes, colds, coughs, bronchitis and heartburn (van Wyk and Wink, 2004). This plant leaves are also traditionally used in Kenya against fever (Muthee *et al.*, 2011).

In Eithiopia, the leaves of *A. afra* are utilized against malaria, abdominal pain and headache (Bekele *et al.*, 2015).

Artemisia arborescens also known as "arborescent mugwort" or "great mugwort" is a species with variable appearance having grey/green to silvery leaves. This silvery shrub is 1 metre in size and indeginous to the Mediterranean region. The popular folktale validates its utilization as a medicine against anti inflammation (Ballero, 2001).

An herbaceous perennial plant *Artemisia argyi* with a creeping rhizome is indigenous to Japan, China and the Eastern region of former Soviet Union. This plant is known as "Gaiyou" in Japan, and "Ai ye" in China. It is used in traditional medicine against kidney, liver and spleen infections (Otsuka, 1992).

Artemisia aucheri is a native Iranian plant and used in the traditional medicine against leishmaniasis (Azadbakht *et al.*, 2003).

The leaves powder of *Artemisia biennis* is used in folk preparations as spices and also used as antiseptics. This plant is broadly used in the North American regions for wound healing, treating sores and chest infections (Kershaw, 2000).

The anthelmintic profile of *Artemisia brevifolia* have been revealed and confirmed that this species eliminate problems related to stomach. Further, the medicinal and economic value of *Artemisia brevifolia* was documented (Gillani *et al.*, 2003). This ethno veterinary medicine is widely used in Pakistan as an anthelmintic plant (Iqbal *et al.*, 2004) and the extract from this plant is used as a vermifuge. Its leaves and inflorescence are ground to form powder which is used for gastric problems (Hayat *et al.*, 2009a)

Reid, (2007) reported the folk utilization of *A. californica* in America. The stem and leaves of this plant were used against menstrual problems, respiratory ailments, cold, cough, asthama and rheumatism.

A perennial slightly aromatic herb, *Artemisia campestris* is prevalent in the Southern Tunisia. This plant is often called as "tgouft" in Tunisia. This plants leaves decoction is broadly utilized in traditional medicine because of having antivenin, anti inflammatory, antimicrobial and anti-rrheumatic possessions (le Floc'h, 1983).

Artemisia cana in folk medicine is employed as spice and also used as an antiseptic (Moermann, 1998). The folk utilization of *Artemisia* species was exposed by Ibrar *et al.*,

(2007) through meetings and discussions with native citizens in Shangla, district of Pakistan.

Hayat *et al.*, (2009a) reported twelve *Artemisia* species that are utilized most popularly among people of Pakistan. They use these plants as medicines, food, fumigants and ornaments. Ashraf *et al.*, (2010) also reported eight *Artemisia* species which are popularly used among local inhabitants as folk medicine in Pakistan.

Artemisia douglasiana "California mugwort" is used in folk medicine and is commonly known as matico in Argentina. Leaves infusion of this plant is generally used against gastrointestinal disorders and to treat peptic ulcers (Ariza-Espinar and Bonzan, 1992).

Artemisia dracunculus with common name "tarragon" is a perennial herb with a long ethnobotanical background. The two well described cultivars of this plant (French and Russian) are extensively utilized and they are differing from each other on the basis of morphology, ploidy level and chemistry. The chemical composition is reported in the literature mainly focused on the composition of essential oil, which gives this plant a unique flavor (Obolskiy *et al.*, 2011).

Leprosy is one of the oldest diseases and in Tangail Bangladesh and leaf decoction of *Atemisia dubia* is utilized to treat leprosy. This plant is also used as fermenting medium (Anisuzzaman *et al.*, 2007).

A. dubia is also employed for asthma, stomachic, ulcers and skin diseases in Nepal (Sapkota, 2008). In Pakistan, the leaf powder of this plant is used for gastric problems and is operative against intestinal worms. Fresh leaves paste of this plant is applied externally for wound treatment and skin related infections (Hayat *et al.*, 2009a).

The stem and leaves decoction of *Artemisia frigida* are used against cough and used to cure diabetes (Gruenwald, 2000).

Artemisia fukudo is distributed in the shorelines of South Korean Jeju Island, South Korean Peninsula, Japan and Taiwan. This plant is employed as a flavouring agent in foods and also used in cosmetic products. This plant also holds anti inflammatory, antibacterial and antitumour efficacies (Lee, 1979).

Artemisia haussknechtii in Iran is used as flavouring in foods. This plant is also used in making perfume products (Zargary, 1997).

Artemisia herba-alba is used in the traditional medicine of Northern Pakistan where its whole plant powder is used against diabetes and its decoction is utilized for cooling purposes. Fumigation with this plant is also effective against muscular pain (Hayat *et al.*, 2009a). In the Northern Badia region of Jordan, decoction of *A. herba-alba* leaves is employed conventionally against fever and menstrual problems. This plant is also used to treat neural diseases (Alzweiri *et al.*, 2011).

Artemisia iwayomogi is a perennial herb and is native to Korea. It is locally called "dowijigi" and "hanin-jin" in Korean. This plant is traditionally used to cure liver ailments predominantly the hepatitis (Park, 1999).

Artemisia japonica locally called as "Kanyarts, Burmar, Basna tashang, and Kharkhaliech" in Pakistan is used in the form of decoction against malaria (Hayat *et al.*, 2009a).

A perennial fragrant shrub *Artemisia judaica* is found extensively in the deserts of Egypt, North African and Middle-Eastern countries, where this plant is used as an anthelmintic drug. Its common Arabic name is "Shih" in the Middle East countries (van Wyk and Wink, 2004).

The inhabitants of Northeastern Mexico use leaves infusion from *Artemisia ludoviciana* against diarrhea (Monroy-Ortiz and Castillo-España, 2007). Some threatened plants were also investigated which were gathered from two different heights in Gilgit-Baltistan Pakistan. Among these plants, *Artemisia laciniata* is employed for the treatment of gall bladder infection, jaundice and high fever.

Artemisia maritima locally called "Zoon, Tarkh and Rooner" in the Northeastern Pakistan is used as anti-inflammatory and antiseptic. Its decoction is used to treat malaria and leaves powder is used against intestinal worms (Hayat *et al.*, 2009a). Additionally, for skin infections, leaf paste of *Artemisia maritiama* is utilized in Pakistan (Fahad and Bano, 2012).

Artemisia nilagirica, The Indian wormwood is found in the mountainous areas of India and alsoutilized as insecticide (Bhattacharjee, 2000).

Artemisia princeps also called "Yomogi" or "Japanese mugwort" is popular in Japan. This plant is significant for the preparation of Japanese confection called kusa-

mochi. It has also been utilized as folk Asian remedy against circulatory disorders, diarrhea and inflammation (Park, 1999).

Artemisia roxburghiana locally called "Garrotra" in Pakistan has been used as traditional medicine in the form of decoction in contradiction of fever. Plant powder is used against intestinal worm (Hayat *et al.*, 2009a).

Artemisia rubripes has been utilized as a haemostatic agent and traditional Korean medicine. It is also used against problems related to stomach including diarrhea and vomiting (Lee, 1979). A study from Chitral region of Pakistan showed the traditional utilization of *Artemisia rutifolia* against gastrointestinal complications. Moreover, stomach worms and fever are also cured with this plant (Ali and Qasir, 2009).

Artemisia santolinifolia locally called the Dron in the North region of Pakistan is employed as folk medicine for the treatment of stomach related problems and intestinal worms (Hayat *et al.*, 2009a).

An annual herb *Artemisia scoparia* "Redstem wormwood" is slightly aromatic and extensively found globally, principally in the central Europe and Southwest Asia. This plant possesses insecticidal, antibacterial, diuretic, anticholesterolemic, antiseptic, antipyretic, cholagogue, vasodilatatory activity and purgative. This plant has been used to treat inflammation of gall bladder, jaundice and hepatitis (Gruenwald, 2000). *Artemisia scoparia*, locally called "Dona, Jhau, Lasaj, Marua, Jaanh Churi-Saroj and Jaukay" in the Northeastern Pakistan is used against burns. An infusion of this plant is utilized as a depurative (Hayat *et al.*, 2009a).

Artemisia tridentata, a big sagebrush is native and a noteworthy shrub found in North American regions. This species is important source of food for many invertebrates and animals (Moermann, 1998). A perennial weed *Artemisia vulgaris* "mugwort" is native to Europe, North America and Asian regions. This plant is locally called "herbaka" and has extensively been utilized as folk medicine in Philippines. This plant is used as an anti-hypertensive remedy. It also holds antispasmodic, anthelmintic, anti-inflammatory and carminative possessions. One special aspect of this plant is to treat dysmenorrhoea and in the induction of labour or miscarriage (Quisumbing, 1978). Leaves paste of *A. vulgaris* commonly called "Nagdowna, Tarkha and Tatwan" in Pakistan is traditionally used to treat fever and its tomentum is used as moxa (Hayat *et al.*, 2009a).

Table 2.1. *Artemisia* species ethno botanically identified in Pakistan.

***Artemisia* Spp.**	**Locality**	**Reference**
A. indica Willd. *A. roxburghiana* Wall. *A. laciniata* Willd.	Shogran valley, Mansahra	Matin *et al.*, 2001
A. brevifolia Wall. ex DC. *A. scoparia* Waldst. & Kit. *A. absinthium* L.	Rawalpindi, Islamabad	Qureshi *et al.*, 2002
A. scoparia Waldst. & Kit. *A. vulgaris* L.	Gokand velly, Buner	Khan, *et al.*, 2003
A. brevifolia Wall. *A. maritima* L.	Kuram agency	Gilani *et al.*, 2003
A. absinthium L. *A. brevifolia* Wall. *A. maritima* L. *A. gmelinii* Web. ex Stechm. *A. stricta* Edgew. *A. tournefortiana* Rchb.	Astor valley, Diamer	Khan, 2004
A. scoparia Waldst. & Kit.	Ghalegay, Swat	Hussain *et al.*, 2006
A.maritia L. *A. absinthium* L.	Gilgit	Qureshi *et al.*, 2006
A. parviflora D. Don. *A. santolinifolia* Turcz. ex Bess.	Booni valley, Chitral	Ahmed *et al.*, 2006
A. roxburghiana Wall. *A. indica* Willd. *A. scoparia* Waldst. & Kit.	Hazara	Rauf *et al.*, 2007
A. flavum *A. jacquemontii* *A. dubia* L.	Ranyal hills, Shangla	Ibrar *et al.*, 2007
A. absinthium L. *A. brevifolia* Wall. *A. scoparia* Waldst. & Kit.	Haramosh and Bugrote valley, Gilgit	Khan and Khatoon, 2008
A. scoparia Waldst. & Kit.	Pindigheb, Attock	Hayat *et al.*, 2008
A. annua L.	Narowal Shakargarh,	Sardar and Khan, 2009

Table 2.2. The ethno pharmacological influence of *Artemisia* species in Pakistan with number of studies reported and number of disease treated by the specific species.

Name of Species	Number of reports	Number of diseases
A. absinthium L.	07	17
A. annua L.	02	08
A. brevifolia Wall.	08	12
A.dracunculus L.	01	01
A. dubia L.	05	13
A. oliveriana J.Gay ex Bess.	01	03
A. japonica Thunb.	04	04
A. macrocephala Jacquem. ex Besser.	01	02
A. kurramensis Qazilb.	09	28
A. moorcroftiana Wall. ex DC.	01	01
A. roxburghiana Wall.	03	06
A. santolinifolia Turcz. ex Bess.	02	03
A. scoparia Waldst. & Kit.	21	62
A. vulgaris L.	07	11

Source: Nadeem *et al.*, 2013

Table 2.3. Reported ethno botanical uses of the *Artemisia* spp. in different regions of the world.

Artemisia Spp.	Part Used	Disease cure/Uses	Country	References
A. absinthium L.	Whole Plant	Fevers, Malaria, Anthelminthic	Pakistan	Qureshi *et al.*, 2006
	Whole plant	Insecticide, Health tonic	Pakistan	Hayat *et al.*, 2009
	Leaves	Gastric problems, Intestinal worms		
	Seeds	Rheumatism, Dental pain relief		
	Aerial part	Aphrodisiac, strengthen men, intestinal parasites, stomachic	Cuba	Volpato *et al.*, 2009
	Floral top	Emetic	Catolina	Bonet *et al.*, 1999
	Whole plant	Malaria, Gastric problem, Intestinal worm, Health tonic	Pakistan	Ashraf *et al.*, 2010
	Leaves and seeds	Rheumatism		
	Shoots	Typhoid, Conceiving pregnancy	Pakistan	Ahmad *et al.*, 2011
	Aerial part	Anthelmintic, Appetizer, Indigestion	Iran	Amiri and Joharchi, 2012
	Whole plant	Obesity, diabetes, liver infections	India	Hassan *et al.*, 2013
	Leaves	Ear diseases	Pakistan	Awan *et al.*, 2013
	Aerial part	Intestine problems, asthma	Algeria	Benarba, 2016
A. afra Var.	Leaves	Abdominal pain, Headache, Malaria	Eithiopia	Bekele *et al.*, 2015
	Leaves	cold, cough, sore throat, influenza, asthma	South Africa	VanWyk and Wink, 2004
	Leaves	Respiratory ailments, constipation, malaria, and wounds	Africa	VanWyk, 2008
	Leaves	Fever	Kenya	Muthee *et al.*, 2011
A. annua L.	Whole plant	Malaria, fever, cough, cold, diarrhea, local perfumes	Pakistan	Hayat *et al.*, 2009
	Aerial parts	Diabetes, blood pressure, dysentery and cough	Pakistan	Jabeen *et al.*, 2015
	Whole plant	Malaria, fever, indigestion, tuberculosis washing for scab, pruritus and mosquito bite	China	Hong *et al.*, 2015
A. biennis Willd.	Leaves	Sores, Wounds, Chest infections	North America	Kershaw, 2000

Table 2.3. Continued...

	Aerial parts	Bronchitis, stomach pain	Algeria	Benarba, 2016
A. campestris L.	Leaves	Intestinal bloating and intestinal parasites	Algeria	Chermat and Gharzouli, 2015
	Aerial parts	Hepatic protector	Catolina	Bonet *et al.*, 1999
A. californica Less.	Stem and leaves	Menstrual problems, respiratory ailments, cold, cough, asthama and rheumatism	America	Reid, 2007
A. dracunculus L.	Leaves	Gastro problems, Fodder for sheep	Pakistan	Hayat *et al.*, 2009
	Whole plant	Toothache, reduce fever, gastrointestinal problems	India	Kumar *et al.*, 2009
	Leaves	Appetizer, Dyspepsia, Anthelmintic, Antacid and Carminative	Iran	Amiri and Joharchi, 2012
A. frigida Willd.	Leaves and roots	Anti-convulsive, Wound healing, tonic, Lung problems and cough	America	Moerman, 1986
A. herba-alba Asso.	Whole plant	Diabetes, muscular pain and used for fire purposes	Pakistan	Hayat *et al.*, 2009
	Whole plant	Gastrointestinal disorders and Jaundice	Jordan	Nawash, 2013
	Leaves	Stomach pain and genital diseases	Algeria	Chermat and Gharzouli, 2015
	Aerial parts	Stomach-ache and ulcer	Algeria	Benarba, 2016
A. indica Willd.	Whole plant	Ear diseases and insecticide	Pakistan	Awan *et al.*, 2013
	Whole plant	Anti-leech and indigestion	Nepal	Balami, 2004
	Leaves	Natural colorant for food	Vietnam	Luu-dam *et al.*, 2016
	Whole plant	Folk medicine	Nepal	Sigdel *et al.*, 2013
	Leaves, buds and flowers	Anthelmintic, gastro-intestinal ailments and boils	Pakistan	Qureshi *et al.*, 2006
A. maritima L.	Leaves and stem	Anti-inflammatory, antiseptic, malaria, intestinal worms	Pakistan	Hayat *et al.*, 2009
	Leaves	Stomach worm	India	Kumar *et al.*, 2009
	Leaves and stem	Skin infections, inflammation and intestinal parasites	Pakistan	Ashraf *et al.*, 2010
	Leaves	Skin infections	Pakistan	Fahad and Bano, 2012
	Aerial part	Abdominal parasite, Antiseptic, Blood purifier and Vermifuge	India	Rana *et al.*, 2014
	Leaves and stem	Stomach pain and fever	Pakistan	Jabeen *et al.*, 2015

Table 2.3. Continued...

	Whole plant	Pain killer, burns and depurative	Pakistan	Hayat *et al.*, 2009
	Shoots	Making brooms, used as thatching material and Fuel purposes	Pakistan	Ahmad *et al.*, 2011
A. scoparia Waldst & Kit.	Leaves	Stomach problems, intestinal worms, indigestion and joint pain	India	Khan *et al.*, 2009
	Whole plant	Antidote, Burns, ENT problems, Fever, Gastric problems and Malaria	Pakistan	Nadeem *et al.*, 2013
	Shoots and Roots	Diabetes, blood pressure, joint pains including gout and rheumatism	Pakistan	Jabeen *et al.*, 2015
A. Stelleriana Bess.	Whole plant	Stomachic, Carminative, Cold, Hair-Tonic and Skin diseases	China	Li, 1973
	Leaves	Carminative and peptic ulcer		Wiart, 2006
A. tridentata Nutt.	Whole plant	Colds, stomach-aches, fevers, pneumonia, laryngitis, tuberculosis, gum, mouth diseases and pulmonary problems	America	Reid, 2007
	Leaves	Anthelminthic and skin diseases	Pakistan	Hamayun, 2007
	Leaves and tomentum	Fever and Moxa	Pakistan	Hayat *et al.*, 2009
	Whole plant	Cardiac problems	Pakistan	Zareen *et al.*, 2013
A. vulgaris L.	Leaves	Natural colorant for food	Vietnam	Luu-dam *et al.*, 2016
	Leaves	To stop nosebleeds, mouth ulcers	Nepal	Gaire and Subedi, 2011
	Flowers	Nerve Tonic, Sexual Impotency and Menstrual Regulator	Iran	Amiri and Joharchi, 2012
	Leaves	Malaria and fever	Pakistan	Ashraf *et al.*, 2010
	Leaves	Malaria and fever	Pakistan	Ashraf *et al.*, 2010
	Leaves	Fever, Gastric problems, Malaria and Vermifuge	Pakistan	Nadeem *et al.*, 2013

2.2. Morphology of *Artemisia*

The inclusions and exclusions of species with in the genus *Artemisia* are continued since many years. In *Artemisia*, phylogenetic relationships for its taxonomic classification remained controversial and it requires a serious and detailed investigations. Its taxonomy is stagnantly a complex and confusing job for several taxonomists because of diversity in hybrid form. Developments in reliable morphological and molecular phylogenetic approaches have stimulated numerous researchers to perform different inquiries about the taxonomy of genus *Artemisia* (Hayat *et al.*, 2009b).

It is well known that the morphology of plants is prominent feature in order to create a phylogenetic tree for better understanding their systematics (Taia, 2005).

Cronquist (1955) studied the detailed phylogeny and taxonomy of the Asteraceae based on morphology. He corroborated the phylogenetic relationships of the Asteraceae family with other allied families and also stated the taxonomic relation on tribal basis like Anthemideae, from where *Artemisia* belongs.

In a study, cladistic investigation of *Artemisia* with its associates was carried out by Jiang and Ling (1992). They primarily focused on morphology of *Artemisia* species and their results were in agreement with postulates of Ling (1982) that worked on relationship of *Artemisia* species along with their classification.

After the effort of Ling (1992), generic monograph of Asteraceae family was distributed by Bremer and Humphries (1993) where they offered a clear scenario of the phylogenetic inquiry of *Artemisia*. They accomplished it by considering the morphological factors.

Bremer (1994) come up with the revised classification and cladistics of the family Asteraceae. He analyzed the position of *Artemisia* on the basis of phylogenetic with the help of morphological traits.

Gustafsson and Bremer (1995) also studied morphology and generated the phylogenetic affinity of the Asteraceae and allied families of the Asterales.

One of the important species is *Artemisia annua*, whose floral morphology was discussed extensively by Ferreira & Janick (1995) for deciphering its taxonomic position.

Another important specie *Artemisia biennis* was studied for the identification and assessment of morphological characters by Kegode and Christoffers (2003). It is quite noticeable that that morphological investigation of fruit along with the seed contributes a pivotal role in systematics studies (Zeng *et al.*, 2004).

Following this, researchers (Kreitschitz and Valles, 2007) inspected the achene and assessed the distribution pattern of slime cells in the achene surface of *Artemisia* species.

In Pakistan, morphology of *Artemisia* genus has been extensively studied by many researchers (Ghafoor, 2002; Abid and Qaiser, 2008; Hayat, 2011). First report of morphology of genus *Artemisia* from Kashmir was given by Kaul and Bakshi (1984).

Another noticeable morphological enquiry on *Artemisia* was specified by Mumtaz *et al.*, 2001. After that the comprehensive morphology of genus *Artemisia* from Pakistan was set by Ghafoor (2002). His work displayed *Artemisia* and *Seriphidium as* detached genus and provided the key for identification of *Artemisia* plants.

Twenty four species of genus *Artemisia* were checked by Abid and Qaiser (2008) suggesting that few micro morphological features of plants are quite important for taxonomic studies.

They primarily worked with the cypsela of *Artemisia* species and explored their taxonomic impact. Moreover, Comprehensive scrutiny of some morphological traits for molecular phylogenies could be fruitful to incorporating the morphological and sequence data together (Scotland *et al.*, 2003).

Additional vital factor to take taxonomic studies is the pollen morphology. Furthermore, with the inventions of scanning electron microscopes, pollen studies have also gained much popularity in deciphering the taxonomy of plants (Taia, 2005).

2.3. Foliar epidermal anatomy of *Artemisia*

2.3.1. Epidermal cells and sotmata

The epidermis possess a lot of important diagnostic characters providing essential clues for the identification of different features including shape, size, stomatal orientation, subsidiary and guard cells, specialized or the distinctive form of trichomes, structural individualities of the walls of epidermal cell (Dickison, 2000).

It is evident from research that the foliar epidermis in plants is very noteworthy taxonomic trait from the biosystematics point of view and also the studies based on taxonomy of many plant families are carried out on the basis of leaf epidermis characteristics (Bhatia, 1984; Jones, 1986). The epidermal anatomy of leaves of many *Artemisia* species have been described by researchers around the world (Naseri, 2004; Rabie *et al*., 2006; Zarin Kamar, 2006; Noorbakhsh *et al*., 2008; Hayat *et al*., 2009)

Nautiyal and Purohit (1980) revealed the pattern of *Artemisia* species on high altitude acclimatization prospects. Their study also disclosed the anatomical changes along with stomatal frequency in the leaves of different *Artemisia* species.

Naseri (2004) in a botanical and ecological study of the species of *Artemisia* in east province of Iran studied anatomical features of seven species of that region. Marchese *et al*. (2005) studied leaf anatomy and carbon isotope composition of the useful herb, *A. annua* L. Zarinkamar (2006) discussed the stomatal types of *Asteraceae* and revealed the stomatal type of *Artemisia* as anomocytic to anisocytic. Rabie *et al*. (2006) also studied five *Artemisia* species in north Iran.

The anatomical examination of 28 species of *Artemisia* for their possible taxonomic applications was done by Noorbakhsh *et al*., (2008). On the basis of foliar anatomy they found three groups within the genus *Artemisia*. They further suggested that using anatomical characteristic is easy to categorize close affinities in species morphological features.

In another study, the taxonomic significance of foliar epidermal cells appearance along with the diversity of stomata in 24 species of *Artemisia* was examined by Hayat *et al*., (2010). They reported novel forms of stomata and also gave detail of differences in epidermal cells, of upper and lower sides of leaves to elucidate taxonomy of *Artemisia*. They found that majority of *Seriphidium* species have lengthened plane walled cells

however other *Artemisia* species have curvy margined unbalanced shape cells with little exemptions. Their inquiry indicated foliar epidermal features as precious traits taxonomically, which may be helpful to resolve taxonomic problems in the genus.

In district Tamil Nadu of India, Rani *et al.*, (2012) worked on the leaf anatomy of *Artemisia nilagirica* and found epidermal cells with wide, wavy and thick anticlinal walls. Their study also revealed anomocytic stomata without distinct subsidiary cells in both adaxial and abaxial surface of the leaves of this plant.

In Poland, Konowalik and Kreitschitz (2012) studied the comparative morphological and anatomical analyses of two varieties of *Artemisia*, i.e. *Artemisia absinthium* var and var. *calcigena* native to the Pieniny mountains of Western Carpathians. They found a thin layer of cuticle in the epidermis of studied *Artemisia* species. They further nticed wavy-shaped outlinein the anticlinal cell walls of the epidermal cells. Stomata were scattered only on the lower leaf surface of both studied species.

Gao *et al.*, (2013) studied the difference in responses of stomatal conductance to moisture stresses between deciduous shrubs and *Artemisia* subshrubs.

In Egypt, an important perennial fragrant small shrub, *Artemisia judaica* L. was characterized for their microscopical features by Bakr (2014) where the epidermal layers were found to be squarish with thick cuticle and was arranged in a single layer.

Srilakshmi and Naidu (2014) studied foliar epidermal features in *Artemisia vulgaris* where the epidermal cells were irregular sinuous with anticlinal walls. They also found anomocytic type stomata in *Artemisia vulgaris*.

In one study, Bano *et al.*, (2015) revealed that the epidermal cells in *Artemisia persica* were irregular/polygonal with straight or undulate walls. Their study also unveiled anisocytic type of stomata in the studied *Artemisia* species.

Ivashchenko and Ivanenko (2017) elaborated the abaxial and adaxial epidermis layer of *Artemisia abrotanum* leaves which were covered with cuticle. They found amphistomatic leaf blades and the stomata were oval anomocytic type.

In one more study, Rahmawati *et al.*, (2017) showed the stomata density and size of *Artemisia annua* in combination with *Gloriosa superba* seeds, which was used as a mutagen. Their results showed increased stomatal size and density with elevated extract concentration.

2.3.2. Foliar Trichomes

The term trichome is derived from "trichos". A Greek word which means hair. In most cases, these trichomes are not connected to plants vascular system but they are extensions of epidermis from which they originate (Wagner, 2004).

Trichomes are present in the upper part of the leaves of many plants which defends the plants against pathogens and herbivores attack (Goertzen and Small, 1993; Shepherd and Wagner, 2007). Trichomes are present in many different forms and shapes that directly hamper feeding of herbivores by acting as a physical barrier to both of their feeding and movement (Chu *et al.*, 2003).

In the species of the genus *Artemisia*, glandular trichomes are the main source where accumulation of many types of secondary metabolites occurs (Slone and Kelsey 1985). The species of *Artemisia* are also rich in sesquiterpenoid lactones (Marco and Barbera 1990), and these are located in the aerial cells of the glandular trichomes of *Artemisia umbelliformis*.

Studies suggest that the morphological traits like floral characters, leaf trichomes and pollen structure are beneficial for taxonomic studies in the genus *Artemisia*.

As the leaf foliar trichomes showed their potency in taxonomy of plants, Many plant systematists and morphologists are greatly attracted to clear the taxonomic controversies (Fang and Fan, 1993). Kelsey (1983) studied the taxonomic status of *Artemisia nova, Artemisia tridentata* and *Artemisia arbuscula* on the basis of both glandular and non-glandular trichomes.

Smith and Kreitner (1983) disclosed the types of leaves trichomes in *Artemisia ludoviciana* Nutt.

Slone and Kelsey (1985) extracted and then purified glandular excretory cells from *Artemisia tridentata* and also enlightened their importance. Ascensao and Pais (1987) studied *Artemisia campertris*. for its glandular trichomes. They also explained the histochemistry and ontogeny of the secretory products.

In another investigation, Lodari *et al.*, (1989) examined 12 *Artemisia* species foliar leaf surface by using scanning electron microscope. Their objective was to authenticate the possibility of identifying species and their close relatedness on the basis of foliar epidermis anatomical attributes.

In a study *Artemisia annua* foliar tissues were assessed that depicted presence of glandular with non-glandular types of trichomes (Duke *et al.*, 1994) and the description of floral characters of *Artemisia annua* with special emphasis to trichomes have also been performed by Ferreira and Janic (1995).

One more investigation also disclosed the foliar structure of *Artemisia annua* and its biochemical study has also been done (Marchese *et al.*, 2005).

Based on the foliar trichomes, Hayat *et al.*, (2009b) described phylogenetic associations of the genus *Artemisia*. They analyzed 24 species data for their phylogenetic study and 8 new kinds of foliar trichomes in genus have been designated. Their investigation discovered foliar trichomes of *Artemisia* as a crucial taxonomic markers and can possibly resolve taxonomic clashes inside the genus.

Bercu and Broască (2011) studied the histoanatomical features of the root, stem and leaf structure of *Artemisia alba* subsp. *saxatilis* (Willd.) and confirmed the presence of both glandular and non-glandular trichomes. Kjær *et al.*, (2012) described the consequences of external stress on both size and density of glandular trichomes in *Artemisia annua*.

In another study using comparative proteomics, Wu *et al.*, (2012) and Bryant *et al.*, (2016) investigated the proteins from glandular trichomes in *Artemisia annua*.

Tan *et al.*, (2015) studied the molecular basis triggering the trichomes development and biosynthesis of artemisinin in *Artemisia annua* L. Rostkowska *et al.*, (2016) explained the accumulation of silicon in the glandular trichomes of *Artemisia annua*. for the production of Artemisinin.

2.4. Pollen Morphology of *Artemisia*

The structure and forms of pollen in the Asteraceae family possess a huge variation, as the defined number of pollen types studied formerly for the family (Jeffrey, 2007). The features of pollen embrace boundless taxonomic worth which has been employed as fruitful phylogenetic markers. For example in sub tribe Artemisiinae, there have been two pollen types recognized on exine ornamentation basis (Stix, 1960). These types comprise the *Anthemis*-type, containing spines (echinate), and the other one is *Artemisia* type containing spinules (microechinate).

Jiang *et al.*, (2005) in their investigation, further stretched the *Artemisia* pollen traits and suggests two groups of *Artemisia* pollen. One is *Mongolica*-type and the other one is *Myriantha*-type, and they are further subdivided in 4 types (*Oligocarpa*, *Sacrorum*, *Anomala* and *Lavandulaefolia*).

The *Artemisia* genus yields ca. 689 pollens per anther (Subba-Reddi and Reddi 1986). Pollen of this diverse genus is quite unique comparatively and is easily distinguishable, because of the presence of miniscule spines or sometimes may not contain spines (Bremer and Humphries, 1993). The sizes of these spines are very tiny but are existing in greater amount (Jiang, 2005).

The pollen morphology of different *Artemisia* species and its systematic implications has been scrutinized by many researchers worldwide (Sing and Joshi 1968; Rowley *et al.*, 1981; Jiang, 2005: Ghahreman *et al.*, 2007; Pellicer *et al.*, 2009 and Hayat *et al.*, 2010). On the basis of pollen morphological data, these studies anticipate that the pollen of *Artemisia is* isopolar, 3-zonocolporate, radially symmetrical, prolate or perprolate and sometimes microechinate.

Few researchers suggests that the pollen features have limited diagnostic potential which may not hold great importance in distinguishing between species (Jiang *et al.*, 2005; Hayat *et al.*, 2010).

In one study, Sing and Joshi (1968) explained the morphology of pollen of some Eurasian *Artemisia* species.

Rowley *et al.*, (1981) investigated the substructure in pollen exines of *Artemisia vulgaris* L. by partial oxidation method with 2-aminoethanol followed by the expansion of exine and its contact to potassium permanganate.

Moreover, a palynological study on *Artemisia*, and its associates were accomplished where with short spinules the ornamentation was considered as a good taxonomic marker for *Artemisia* and its associates (Martin *et al.*, 2003).

Hodin *et al.*, (2005) examined the release of some nondiffusional allergens from pollen grains of *Artemisia vulgaris*.

In another study, 26 species of *Artemisia* from Iran have been inspected with light and scanning electron microscope with emphasis to the morphology of pollen (Ghahreman *et al.*, 2007). Pellicer *et al.*, (2009) investigated *Artemisia* pollen type comparison to *Ajania* where the shape of *Artemisia* pollen found was spherical, and in some cases slightly oblate or prolate.

Hayat *et al.*, (2010) gave a quantitative explanations using both light microscopy and scanning electron microscopy in the pollen of different *Artemisia* species. They drew a phylogenetic tree which authenticated that sect. *Artemisia* and sec. *Seriphidium* are monophyletic.

Malkiewicz *et al.*, (2014) studied *Artemisia* pollen on weather conditions basis in Wrocław Poland.

Depciuch *et al.*, (2016) analyzed the pollen of *Artemisia vulgaris* on the basis of molecular composition and morphological changes traffic pollution with the help of FTIR spectroscopy and SEM.

Bogawski *et al.*, (2016) suggests that the *Artemisia* pollen is a noteworthy allergen in Europe. They scrutinized three *Artemisia* species for their potential pollen emission and flowering phenology in Poznan (Western Poland).

2.5. Phytogeography of *Artemisia*

The geographical pattern of *Artemisia* has been investigated by a number of researchers (Ghafoor, 2002; Tkach *et al.*, 2007).

Ling *et al.*, (2006) documented *Artemisia* distribution predominantly in China and other nations where *Artemisia* has been documented. The temporal and spatial progression and the connection of *Artemisia* with Tibetan Plateau elevation were checked (Yunfa *et al.*, 2011). They have done this by assembling data of pollen from 122 places across China. Their data presented that in the late Eocene, *Artemisia* may be initiated from arid or the semi-arid latitudes of Asia and due to the initial elevation of Tibetan Plateau and the worldwide cooling, *Artemisia* may be dispersed east and west in the Oligocene.

In another study, a sketch of the distribution of *Artemisia* has been presented (Tkach *et al.*, 2007). They separated the world into 12 floristic areas where the multiplicity of *Artemisia* occurs. According to them, Pakistan resides in the floristic region of South West Asia. Stewart (1972) presented a rough sketch of *Artemisia* distribution in Pakistan and tried to enlist the areas where *Artemisia* have been found.

For the flora of Pakistan, Ghafoor (2002) documented the regions from where the herbarium samples of *Artemisia* have been assembled. On the other hand, three phytogeographic regions in Pakistan like, Irano-Turanian, Indian and Saharo-Sindian were given by Takhtajan (1969) and Good (1974). Later on Ali and Qaiser (1986) proposed the diversity and distribution of flowering plants in Pakistan by dividing the country into four phytogeographical regions namely Irano-Turanian, Sino-Japanese,Indian and Saharo-Sindian. They again divided whole Pakistan into five floristic divisions (Hayat, 2011) as shown in figure 2.1, and these divisions are basically more appropriate for investigators.

One study was undertaken to resolve the relationships within species of *Tridentatae* and their close associates by delineating their phylogenetic affinities (Garcia *et al.*, 2011). They proposed a new limitation and divided the subgenus into 3 sections namely, *Filifoliae*, *Tridentatae*, and *Nebulosae*. The position of the circumboreal and other North American species suggests that *Artemisia* is inherited store for the New World endemics, together with South American native.

Artemisia phylogenetics biogeographically in context to the Beringian Region was investigated by Riggins and Seigler, (2012). They employed ITS phylogeny contained 173

accessions depicted the paraphyletic nature of *Artemisia*. Their study excluded numerous small Asian genus and also the *Sphaeromeria* which is genus from North America. They also revealed that *Artemisia* from North America has numerous ancestries, and that western North America has helped for the colonizing in Southern America and eastern Asia.

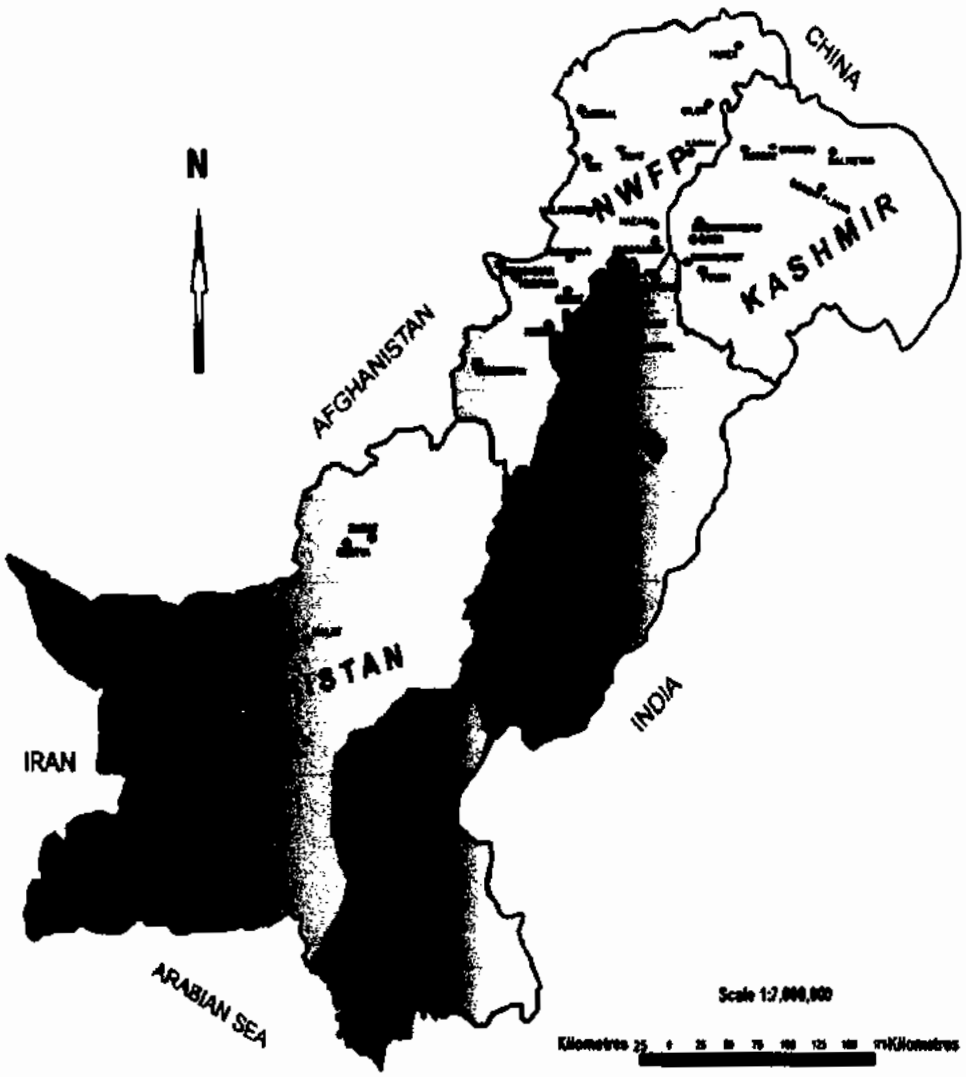

Figure 2.1. Major localities (Black dots) of *Artemisia* in regions of Pakistan (colored zones). Picture adapted from Hayat, (2011).

2.6. Molecular Phylogeny of *Artemisia*

Other than the morphological data, DNA regions in *Artemisia*, like internal transcribed spacer (ITS), external transcribed spacer (ETS) of nuclear ribosomal DNA (nrDNA) and chloroplast DNA (cpDNA) regions have also been broadly employed for phylogenetic revisions (Kornkven *et al.*, 1999; Torrel *et al.*, 1999; Tkach *et al.*, 2007; Sanz *et al.*, 2008). However, for phylogenetic reconstructions the combination of two regions gives the basis of important data, and can hypothesize supplementary enquiry. There exist more techniques that may quite efficient in resolving the evolution question based on plant genomic studies i.e. FISH (fluorescent in situ hybridisation) with rDNA probes and genome size estimation (Lim *et al.*, 2007).

Linder *et al.*, (1999) suggests that there are DNA regions which are needed for molecular phylogenetic restoration of various plants. These regions evolve rapidly than the internal transcribed spacer (ITS), which is repeat of 18S-26S nuclear ribosomal DNA. They proposed a technique for ETS amplification and sequencing for Asteraceae and some narrowly associated families. These methods in genus *Artemisia* gives extraordinary understandings for its organization and also provides facts about the genomic level changes (Hoshi *et al.*, 2006).

In a phylogenetic analysis, the ITS sequences noticeably validated the monophyletic nature of *Artemisia* in broader sense, i.e. with the five main assemblies, with subgenus *Tridentatae* and subgenus *Seriphidium*. These two major subgenera in genus *Artemisia* have homogamous capitula, but these studies were authenticated by plaenological and carpological attributes (Torrell *et al.*, 1999).

The phylogenetic study for the subtribe Artemisiinae of Anthemideae tribe was performed where the nuclear ribosomal DNA regions like internal transcribed spacers (ITS) were utilized (Watson *et al.*, 2002). Their final phylogenetic tree showed 3 major clades that represents the radiate genera (*Dendranthema* and *Arctanthemum*), and tree was also accompanied with two clades of *Artemisia* genus.

Another study investigated internal transcribed spacer and external transcribed spacers (ITS1 and ITS2) sequences of nrDNA for forty four (44) species of *Artemisia* that signifies geographical range and the five classical sub genera of the genus (Valles *et al.*, 2003). They observed that 11 species other than 10 genera are attentively connected to

Artemisia and 6 outgroup species from 5 other genera of the Anthemideae. Their results support the monophyletic nature of *Artemisia* genus.

Based on ITS and ETS sequences, a phylogenetic tree was created, whereas in many details, merely topology failed to bolster the traditional classification. e.g., *Seriphidium* is demonstrated two autonomous groups and 3 among the 4 sections inspected in *Artemisia* subgenus were found to be polyphyletic (Tkach *et al.*, 2007).

In order to understand the evolutionary history of *Artemisia* and its relationships with other genera of subtribes *Artemisiinae, Leucantheminae* and *Tanacetinae*, Sanz *et al.*, (2008) newly generated 63 ETS and 10 ITS sequences of nrDNA in *Artemisia*. Their analysis was done on the combined dataset using maximum likelihood, maximum parsimony and Bayesian inference. Their combined analysis supports that all *Artemisiinae* genera including *Hippolytia* and *Nipponanthemum* constitute a monophyletic group. They concluded that *Artemisia/Kaschgaria* lineage probably originated from an ancestor with disciform capitula, central hermaphrodite florets and *Artemisia* pollen type.

In one more study sequences from nuclear DNA were used to untie the interspecific associations among the *Artemisia* of South America and their associates with other unsettled species of the genus, also using FISH and genome size calculations to characterize this polyploidy level (Pellicer *et al.*, 2010). They found a monophyletic clade where most species were nested within the American endemic clade, by excluding *Artemisia magellanica*. It seemed separated from American species and establishes a clade organized with *Artemisia biennis*. Analysis of FISH and the data of genome size exposed that monoploid genome size leftovers persistent over ploidy levels and they detected increased ribosomal loci, a vigorous not usually found in this genus.

Recently, there have been many studies conducted achieve better understanding and to unveil the close affinities genus *Artemisia* like *Absinthium, Seriphidium* Besser ex Less., *Dracunculus* (Besser) Rydb, and *Tridentatae* (Rydb).

In Pakistan, Hayat, (2011) conducted a phylogenetic study on Pakistani *Artemisia* using ITS and ETS sequences of nrDNA. In his comprehensive study, the reunion of subgenus *Seriphidium* and polyphyletic nature of subgenus *Artemisia* was revealed.

Mahmood *et al.*, (2011) studied *Artemisia* species collected from different localities of Pakistan for the determination of phylogenetic relationship. These studies were performed using the RPS11 region of chloroplast DNA.

In another study, Hawaiian *Artemisia* was scrutinized for the molecular phylogenetic studies and worldwide divergence on the basis of chloroplast DNA and nuclear markers by Hobbs and Baldwin (2013).

For the reinvestigation of the phylogenetic relationships in *Artemisia*, ITS and *psbA-trnH* sequences within 3 subgenera of *Artemisia* (subgenus *Dracunculus*, subgenus *Artemisia*, and subgenus *Serphidium*) were studied (Haghighi *et al.*, (2014). Their results disclosed that the three subgenera were seperated from each other, where the heterogamous subgenus *Artemisia* and subgenus *Dracunculus* are closely associated to each other than to the homogamous subgenus *Serphidium*. They proposed that *Artemisia* comprises of several emerging subgenera.

In their recent study, Koloren *et al.*, (2016) phylogenetically analyzed the internal transcribed spacer (ITS) nucleotide sequences of 18S-26S rDNA, of 19 *Artemisia* species from the Ordu Province Turkey. Their study revealed two unique haplotypes with in the studied *Artemisia* samples i.e., Haplotype-I and Haplotype-II. These haplotypes were appeared with the lineage same as *Artemisia argyi*, *Artemisia sylvatica* and *Artemisia verlotiorum*.

Malik *et al.*, (2017) studied phylogeny of *Artemisia* and subgenus *Seriphidium* species to unveil the taxonomic status using both nuclear and plastid DNA sequences. Their results revealed that the subgenus *Seriphidium* is monophyletic and it is merged into two main clades.

CHAPTER 3

3. MATERIAL AND METHOD

The overall design of present work is given in Figure 3.1.

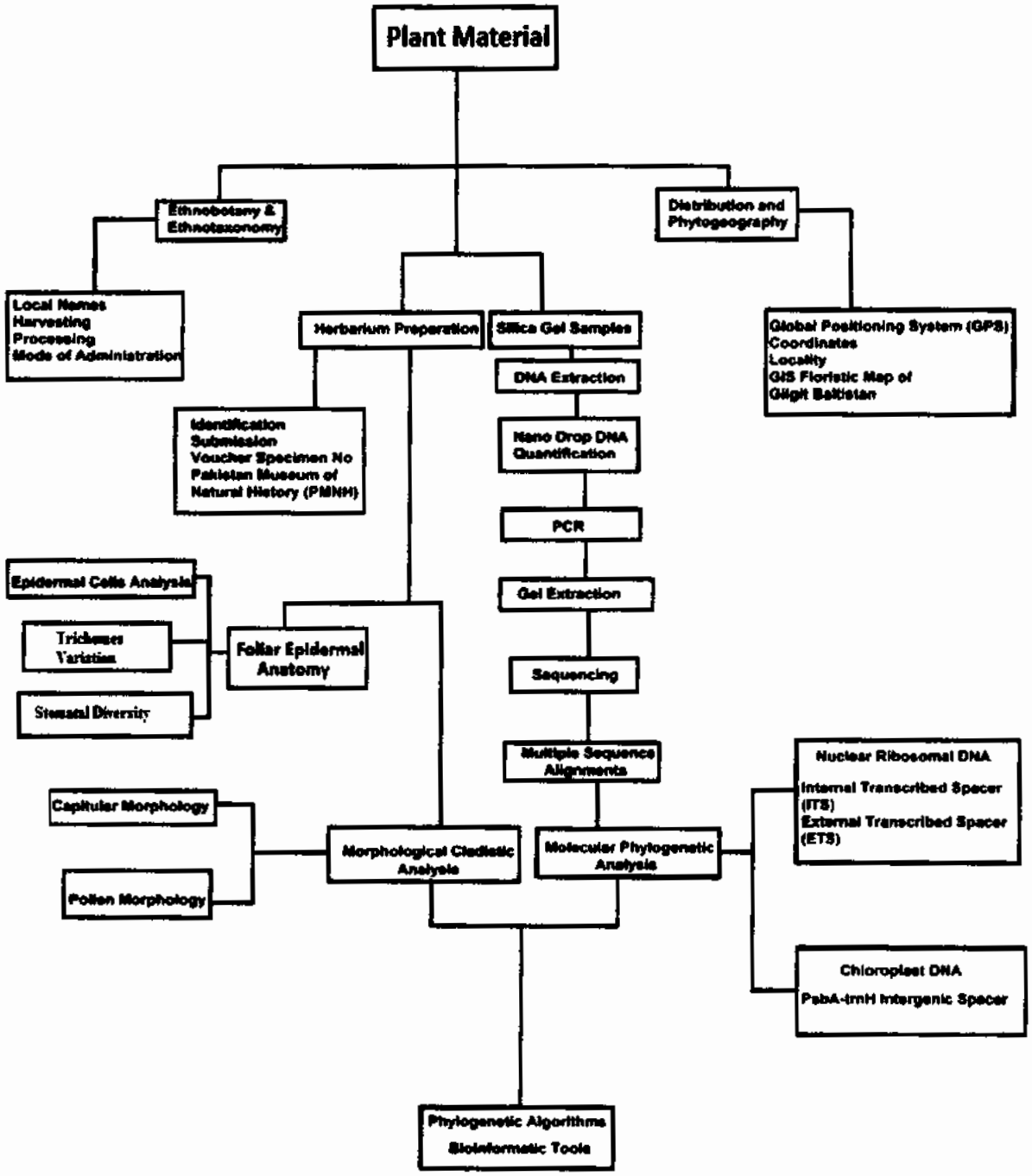

Figure 3.1. Schematic demonstration of the present study

3.1. Plant Material

The samples belong to *Artemisia* species investigated in the present study were obtained from their natural habitat accompanying extensive field visits from different localities of Gilgit-Baltistan region of Pakistan. The particulars of source and collection along with the GPS data of the investigated *Artemisia* species are provided in Table 3.1.

Broad field surveys were scheduled during summer (2016-2017) in different areas of Gilgit-Baltistan where *Artemisia* was formerly reported (Ghafoor 2002; Hayat *et al.*, 2009). Field methods were taken from the litrerature (Martin, 2003; Vogl *et al.*, 2004; Bridges and Lau, 2006) and *Artemisia* species were collected from different regions of five districts of Gilgit-Baltistan. The surveyed regions belong to district Gilgit, Hunza Nagar, Ghizer, Astore and Skardu respectively as shown in Figure 3.2.

3.2. Ethnobotany

3.2.1. *Artemisia* species and Iconography

Primary data have been recorded on the spot during interviews with local people from five districts of Gilgit-Baltistan, and later, a check list of collected *Artemisia* plants was prepared along with photographs to assist the interviews as described by Rehman *et al.*, (2015).

3.2.2. Interviews and Conversations

A questionnaire (Appendix-1) was used to interview the native people during the field surveys and ethnobotanical information was assembled on *Artemisia* species which were normally used in studied localities. Prior to conduct the interviews, the local community was informed and their permissions were received in the form of prior informed consent (PIC). The local names of plants, picking and handling methods and mode of administration against certain ailments were noted through the questionnaire as described by Hayat *et al.*, (2009).

The conversation was done in local languages (Urdu, Shina, Brushuski, Astori, Khwaar and Balti). A total of 195 key informants (72.50% men and 27.50% women) between the ages of 35 to 60 years were interviewed especially from those having good knowledge about the plants utilization. Interviews were conducted in working days of some common areas like gardens, farms, nurseries, bazars, homes, schools and colleges.

The male informants were mostly farmers, herbalists, shopkeepers, government employees and laborers while the female informants were house wives andfield workers.

3.2.3. Herbarium preparation and plant identification

The plants collected were pressed, dried, labelled and mounted on herbarium sheets for depositing in the Herbarium of Pakistan Museum of Natural History (PMNH) to obtain voucher number for future reference (Table 3.1). The plants were tentatively identified by assessing different morphological characteristics and relating with the samples already existed in the herbarium and consulting the Flora of Pakistan given by Ali and Qaiser, (1993-2010).

Table 3.1. Collection details of *Artemisia* species from Gilgit-Baltistan region of Pakistan with latitude, longitude, location and voucher specimen number.

Taxon	Latitude	Longitude	Location	Voucher specimen no
A. Absinthium L.	N-36'19.756	E-74'52.520	Ataabad Hunza-Nagar	PMNH- 41647
A. annua L.	N-35'54.949	E-74'18.508	Barmas paen Gilgit	PMNH- 41582
A. Arborescens (Vaill.) L. *	N-35'26.758	E-74'47.990	Hacho paen Astore	PMNH- 41702
A. argyi H.Lév. & Vaniot. *	N-35'54.951	E-74'18.503	Barmas paen Gilgit	PMNH- 41583
A. austriaca Jacq. *	N-36'01.609	E-74'33.255	Bagrote valley Gilgit	PMNH- 41643
A. biennis Willd	N-36'09.387	E-74'11.941	Naltar valley Gilgit	PMNH- 41622
A. campestris L.	N-36'08.708	E-74'12.397	Naltar valley Gilgit	PMNH- 41619
A. chamaemelifolia Vill. *	N-36'09.622	E-74'11.622	Naltar valley Gilgit	PMNH- 41630
A. chinensis L. *	N-35'26.585	E-75'27.011	Shangrilla Skardu	PMNH- 41722
A. capillaris L.	N-35'54.503	E-74'23.880	Danyore Oshkandas Gilgit	PMNH-41607
A. dubia L.	N-35'54.491	E-74'23.867	Danyore Oshkandas Gilgit	PMNH- 41608
A. gmelinii Weber ex Stech.	N-36'08.967	E-74'12.112	Naltar valley Gilgit	PMNH-41621
A. herba-alba Asso.	N-35'54.061	E-74'12.762	Kargah nala Gilgit	PMNH-41599
A. indica Willd.	N-36'15.250	E-73'24.240	Yasin Ghizer	PMNH-41694
A. maritima L. Ex Hook f	N-35'56.694	E-74'30.184	Bagrote valley Gilgit	PMNH- 41639
A. montana Pamp. *	N-35'30.883	E-75'40.115	Hashupi Shigar Skardu	PMNH- 41708
A. pontica L. *	N-36'02.121	E-74'35.227	Bagrote valley Gilgit	PMNH-41642
A. rutifolia Var.	N-36'08.708	E74'12.397	Naltar valley Gilgit	PMNH- 41618
A. rutifolia sub sp. *	N-35'11.963	E-75'37.387	Sadpara lake Skardu	PMNH- 41712
A. scoparia Waldst. & Kit.*	N-35'26.665	E-75'26.960	Kachura lake Skardu	PMNH- 41714
A. sieberi Bess.*	N-35'54.785	E-74'18.591	Barmas bala Gilgit	PMNH-41591
A. sieversiana Ehrhl. Ex Willd.	N-36'14.875	E-73'21.810	Khalti lake Ghizer	PMNH-41691
A. tournefortiana Rachb.	N-35'25.493	E-75'44.507	Shigar valley Skardu	PMNH-41704
A. verlotiorum Lamotte.*	N-36'08.543	E-73'51.721	Bubar Ghizer	PMNH-41684
A. vulgaris L.	N-36'20.508	E-74'52.277	Shishkat Hunza-Nagar	PMNH-41646
A. sp. -AD-H**	N-35'55.133	E-74'18.487	Barmas paen Gilgit	PMNH- 41586
A. sp.- A**	N-36'09.612	E-74'12.042	Naltar valley Gilgit	PMNH- 41631
A. sp.- B**	N-36'09.122	E-74'12.045	Naltar valley Gilgit	PMNH- 41632
A. sp.- C**	N-36'20.550	E-74'51.278	Gojal Shishkat Hunza-Nagar	PMNH- 41649
A. sp.- D **	N-36'07.436	E-73'52.341	Thingdas Ghizer	PMNH- 41680
A. sp.- E **	N-35'25.463	E-75'44.366	Shigar valley Skardu	PMNH-41707
A. sp.- F **	N-35'52.680	E-74'26.123	Minawar Gilgit	PMNH-41614
A. sp.- G **	N-35'16.062	E-75'38.045	Manthal village Skardu	PMNH- 41710
A. sp.- H **	N-35'26.764	E-74'47.998	Hacho paen Astore	PMNH- 41700
A. sp.- I **	N-35'54.012	E-74'12.762	Kargah nala Gilgit	PMNH-41602

The voucher numbers have been obtained from Pakistan Museum of Natural History (PMNH). Collectors: Adil Hussain, Tanseer Hussain, Tabeer Hussain and Amar Abbas. "*" Represents rare *Artemisia* species. "**" Represents undescribed *Artemisia* taxa reported first time in this study from Northeast (Gilgit-Baltistan) region of Pakistan

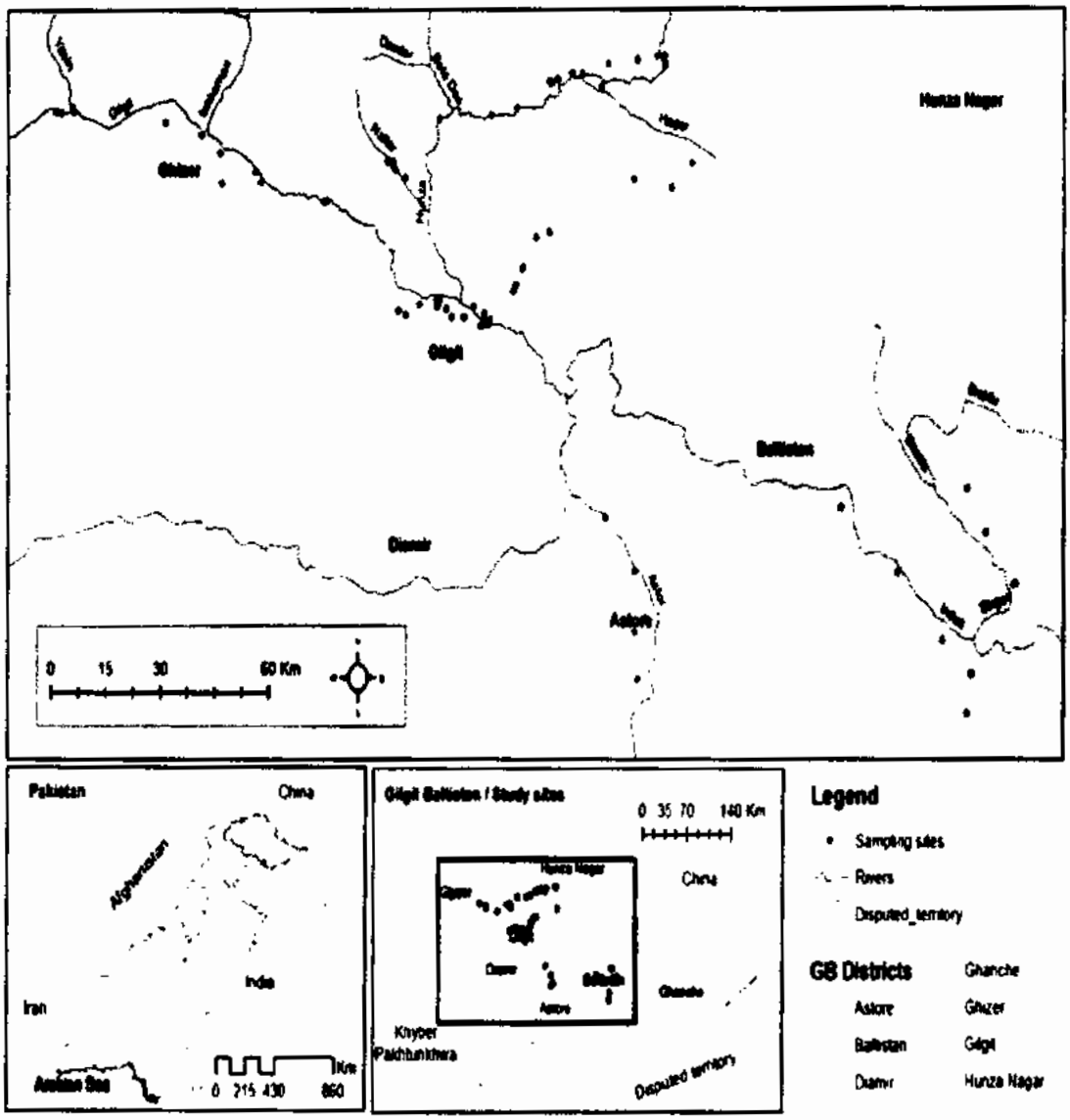

Figure 3.2. Map showing the study area of Gilgit-Baltistan region, Pakistan.
Red dots indicate samples of *Artemisia* from different regions of five districts of Gilgit-Baltistan.

3.3. Morphology of *Artemisia*

The various morphological characters of collected *Artemisia* species listed in Table 3.2 were measured with hard ruler under a dissecting microscope. Magnifiers of 4X, 10X and 20X were also used for observations of various parts.

Flowers, leaves and cypselas were soaked in hot water before dissection. Observations and measurements were made 10 to 15 times for 4-5 accessions of same species in order to ensure the readings.

The resultant data has been arranged in the standard format of taxonomic studies. Graphs of selected morphological traits, showing variations in morphological characters are also given. Representative photographs of examined specimens were presented in the form of Plates.

The morphological characters were further confirmed and authenticated with the help of previous studies conducted in the area by Hayat *et al.*, (2009), Ghafoor (2002), Kaul and Bakshi (1984), Mumtaz *et al.*, (2001) and Abid and Qaiser (2008) for phylogenetic analysis.

On the bases of morphological observations, 66 morphological characters were nominated as a character states for the cladistics analysis of *Artemisia* (Table 3.2).

To unveil the infraspecific diversity of the morphological traits, the continuous character states are considered in order of their degenerative appearance.

The apomorphic or plesiomorphic state of each character was recorded by using the method given by Cronquist (1955).

For the cladistics analysis, *Anthemis arvensis* L. was used as outgroup for assessment based on morphological characters.

An original data matrix was generated with the outgroup assessment method given by Watrous and Wheeler, (1981).

For the cladistic analysis, the generated original data matrix on the basis of morphological characters of *Artemisia* was converted into binary data matrix with the help of FACTOR program of PHYLIP software version 3.67 (Felsenstein, 2007).

The most parsimonious trees (MPTs) on the basis of binary matrix were generated with MIX program of PHYLIP software using the method of Wagner parsimony (Farris, 1970).

A strict consensus cladogram of the MPTs was generated with CONSENSE program of PHYLIP software (Sokal and Rohlf, 1981).

Table 3.2. Morphological characters and character states of *Artemisia* species for the cladistic analysis. Numbers shown in brackets implies the codes for character states. 0 is always the code for plesiomorphic (Ancestral) character state.

Sr.No	Character	Character States
1	Life cycle	Perennial (0), Biannual (1), Annual (2)
2	Life form	Herb (0), Shrubby (1)
3	Occurrence	Common (0), Rare (1)
4	Aromatic	Highly (0), Slightly (1)
5	Habitation	Moist places (0), Dry places (1)
6	Rootstock	Horizontal (0), Vertical (1)
7	Rhizomes	Absent (0), Present (1)
8	Root hairs	Glabrous (0), Hairy (1)
9	Root colour	Creamish (0), Dark brownish (1)
10	Stem hairs	Hairy (0), Glabrous (1)
11	Stem branching	Present (0), Absent (1)
12	Stem glands	Present (0), Absent (1)
13	Stem groves	Slender (0), Sulcate (1), Striate (2), Costate (3)
14	Stem height	10~40cm (0), 20~80cm (1), 25~100cm(2), 30~200cm (3)
15	Stem colour	Greenish (0), Whitish (1), Violet (2), Yellowish (3), Brownish (4), Reddish (5), Purplish (6)
16	Stem lines	Present (0), Absent (1)
17	Woody stem base	Absent (0), Present (1)
18	Branches from base	Absent (0), Present (1)
19	Basal leaf Petiole	Present (0), Absent (1)
20	Basal leaf petiole length	3~10cm (0), 1~2cm (1), <1cm (2), Sessile (3)
21	Basal leaf lamina shape	Ovate (0), Lanceolate (1)
22	Basal leaf upper surface	Hairy (0), Glabrous (1)
23	Basal leaf lower surface	Tomentose (0), Sparsely hairs (1), Glabrous (2)
24	Basal leaf upper surface colour	Green (0), Dark green (1)
25	Basal leaf lower surface colour	Light green (0), Grayish white (1)
26	Basal leaf dissections	Undivided (0), Pinnatifid (1), Pinnatisect (2)
27	Basal leaf lobes shape	Elliptic or ovate (0) Oblong or lanceolate (1)
28	Middle leaf petiole	Present (0), Absent (1)
29	Middle leaf shape	Ovate (0), Lanceolate (1)
30	Upper leaf shape	Lanceolate (0), Linear or filiform (1)
31	Upper leaf margin	Pinnatifid (0), Entire (1)
32	Upper leaf petiole	Absent (0), Present (1)
33	Capitulum shape	Hemispherical (0), Ovoid (1), Glubose (2), Oblong (3)

Table. 3.2. Continued…

34	Capitulum length	>5mm (0), 3~4mm (1), 1~2mm (2)
35	Capitulum width	>4mm (0), 3~4mm (1), 2~3mm (2), 1~2mm (3)
36	Capitulumpedunculate	Present (0), Absent (1)
37	No. of bracts seriate	≥5 (0), 4 (1), 4~3 (2), <3 (3)
38	Outer phyllaries texture	Canescent (0), Slightly hairy (1), Glabrous (2)
39	Outer phyllaries margins	Ciliate (0), Membranous (1), Scarious (2)
40	Outer phyllaries shape	Linear or oblong (0), Ovate (1)
41	Inner phyllaries texture	Canescent (0), Glabrous (1)
42	Inner phyllaries margins	Membranous (0), Scarious (1)
43	Inner phyllaries shape	Ovate (0), Oblong or elliptic (1)
44	Receptacle	Present (0), Absent (1)
45	Receptacle shape	Flattened (0), Convex (1), Hemispherical (2), Conical (3)
46	Receptacle texture	Hairy (0), Glabrous (1)
47	Receptacle diameter	≥2mm (0) 1~2mm (1), <1mm (2)
48	Receptacle colour	Greenish (0), Whitish (1), silver (2)
49	No. of ray florets	>15 (0), 11~15 (1), 6~10 (2), ≤5 (3) Absent (4)
50	Ray florets corolla length	≥2mm (0)1~<2mm (1), <1mm (2) Absent (3)
51	Ray florets corolla shape	Tubular (0), Filiform (1), Urceolate (2), Compressed (3), Absent (4)
52	Ray florets corolla colour	Yellow (0), Brown (1), Greenish (2), Purplish (3), Absent (4)
53	Ray florets	Female (0), Absent (1)
54	No. of disc florets	>40 (0), 31~40 (1), 21~30 (2), 11~20 (3), <10 (4)
55	Disc florets corolla length	>2mm (0), >1~<2mm (1), ≤1mm (2)
56	Disc florets corolla shape	Tubular (0), Clavate (1), Conical (2), Companulate (3)
57	Disc florets corolla colour	Pale (0), Red tinged (1)
58	Disc florets fertility	Bisexual (0), Staminate (1)
59	Cypsela shape	Oblanceolate (0), Oblong (1), Terete (2)
60	Cypsela attachment	Terminal (0), Lateral (1), Oblique (2)
61	Cypsela texture	Striate (0), Glabrous (1)
62	Cypsela Colour	Light brown (0), Dark brown (1)
63	Cypsela length	<1mm (0), ≥1mm (1)
64	Cypsela width	<0.5mm (0), ≥0.5mm (1)
65	Pappus	Absent (0), Present (1)
66	Pappus shape	Absent (0), Glabrous (1), Hairy(2)

3.4. Foliar Epidermal Anatomy

The particulars of source and collection along with the GPS data of the investigated *Artemisia* species for their foliar epidermal anatomy are provided in Table 3.1. The taxonomically essential epidermal attributes (Epidermis cells, stomata and trichomes types) of *Artemisia* species collected from Gilgit-Baltistan region of Pakistan were assessed by means of light microscopy (LM) and scanning electron microscopy (SEM).

3.4.1. Light microscopy (LM)

For light microscopy, dried specimen of each plant from herbarium was taken. Primarily, 30% nitric acid and about 1.5 g of potassium chloride was taken in a test tube, the leaves were boiled for few minutes (2-3 min) in the solution. After boiling, the leaves were washed with deionized water. Peeling of epidermis was performed and 60% potassium hydroxide solution was used to keep the peel for 2 hours. Finally, these peels were transferred to lactic acid and glass slides were prepared for LM investigation (Hayat *et al.*, 2009b; 2010)

3.4.2. Scanning electron microscopy (SEM)

From the dried herbarium specimen, 4-5 mm of top and bottom leaf was taken and then it was fixed in glutaraldehyde (6%) for 24 hours with 0.05 M sodium cacodylate. After that, the samples were rinsed in distilled H_2O for 2-3 times with the help of pasture pipette. After each rinse, ethanol (10-100%) was used to dehydrate the samples for 20 minutes. The samples were then stored in 100% ethanol in the refrigerator for further use. Each dried sample belong to species of *Artemisia* was mounted on aluminium specimen stub with double-sided carbon. Sputter coating was carried out with gold in a sputtering chamber of Pelco Auto sputter Coater (SC-7, Ted Pella Inc).

Both the adaxial and abaxial surfaces of the leaves were examined at different magnifications with Philips XL30 TMP (FEI Company) scanning electron microscope, activated at 10-20 kV of voltage, at the electron microscopy core laboratory, Tupper Hall, University of California Davis, USA. All the demonstrative structures observed were taken digitally with the help of Microsoft image programmed for windows. The method given by Hayat *et al.*, (2010) and Dilcher (1974) was used for the identification of epidermal cells and stomata.

3.5. Pollen Morphology

3.5.1. Pollen Material

The pollen material used in the present investigation was taken from herbarium specimens containing flowers and also from the freshly obtained samples from different regions of Gilgit-Baltistan Pakistan. The details and origin of studied *Artemisia* species have been provided in Table 3.1. Primarily, the pollen grains of 22 *Artemisia* species were prepared for scanning electron microscopy (SEM) by standard methods, described by Erdtman, (1952) and modified by Perveen and Qaiser (2010), Bibi *et al.*, (2008) and Hayat *et al.*, (2010). In order to separate the pollen grains from anthers, stereo microscope was used.

3.5.2. SEM and LM

For SEM analysis, the pollen grains were acetolysed first and then transferred directly to the sticky carbon disc on metal stub. After transferring the pollen on stub, coating with gold in a sputtering chamber was done in Pelco Auto sputter Coater SC-7 (Ted Pella Inc). Philips XL30 TMP (FEI Company) Scanning electron microscope was activated at 5/10 and 20 kV, at the electron microscopy core laboratory, Tupper Hall, University of California Davis, USA.

Micromorphological data of pollen grains was recorded with OLYMPUS/BX-51 light microscope at the Department of Plant sciences University of California Davis, USA. Observations for polar diameter (P), equatorial diameter (E) and ratio of P/E were recorded according to Reitssma (1970).

3.5.3. Cladistic and cluster Analysis

From the data obtained from micromorphological characteristics of pollen in *Artemisia*, an original data matrix (Table 3.3) was produced. This data matrix was then subjected to cladistics and cluster analysis. For cladistic analysis the original data matrix was converted into binary data matrix with FACTOR program of PHYLIP software version 3.67 (Felsenstein, 2007).

The most parsimonious trees (MPTs) based on the binary matrix were generatedwith MIX program of PHYLIP using Wagner parsimony method (Farris, 1970).

Cluster examination was done usingUPGMA method selecting EUCLIDEAN option with MVSP software (Kovach, 2007).

Table 3.3. Pollen characters and character states for cladogram and cluster analysis of *Artemisia*. Numbers inside brackets shows the character state codes. The code of plesiomorphic character (Ancestral character) is always 0.

Character	**Character states**
Pollen type	*Anthemis* (0), *Artemisia* (1)
Pollen shape	Globular (0), Oblate (1)
Spinules arrangement	Dense (0), Loose (1)
Exine sculpture	Granular (0), Sinuolate (1)
Spinules base	Stretching and outward extending (0) Normal* (1)
Polar length	> 26µm (0), > 23-26µm (1), > 22-23µm (2), > 21-22µm (3), > 20-21µm (4), > 19-20µm (5), > 18-19µm (6), > 17-18µm (7), 16-17µm (8), 15-16µm (9)
Equatorial width	> 21µm (0), > 20-21µm (1), > 19-20µm (2), > 18-19µm (3), > 17-18µm (4), > 16-17µm (5), > 15-16µm (6) 14-15µm (7)

3.6. Phytogeography

Global positioning system (GPS) data of their vicinities was appropriately recorded for the collected samples. The comprehensive facts of all the *Artemisia* species (Name, GPS readings of its localities) was upheld in a Microsoft® Excel spread sheets and employed for GIS representation by means of ArcGIS® version 9.1. GIS layers used in this study comprise floristic partitions of Gilgit-Baltistan, localities data. The floristic diagram of Gilgit-Baltistan was based on Ali and Qaiser (1986) phytogeographical areas.

3.7. Molecular Phylogeny

Plant material used for the molecular phylogeny was taken from herbarium specimens and also from silica gel dried samples which were obtained during the survey to different vicinities of Gilgit-Baltistan region of Pakistan. These plant samples represent all sub genera of genus *Artemisia.* The origin and detail of studied *Artemisia* species is provided in Table 3.1.

In order to generate a better evolutionary context, in which to circumscribe *Artemisia* species from the Gilgit-Baltistan region of Pakistan, a comprehensive phylogenetic reconstruction of *Artemisia* has been performed. Specifically, previously published internal transcribed spacer (ITS), external transcribed spacer (ETS) of nuclear ribosomal DNA (nrDNA) and Chloroplast *psbA-trnH* sequences of taxa representing all subgenera of *Artemisia* were retrieved from Genbank (Appendix 2).

Twenty-Eight species of *Artemisia* collected from different regions of Gilgit-Baltistan Pakistan were included from five subgenera of genus *Artemisia*, including subgenera *Artemisia*, *Absinthium*, *Dracunculus*, *Pacifica* and *Serphidium* and all the collected *Artemisia* species were newly sequenced. *Chrysanthemum indicum*, *Chryasanthemum mongolicum and Ajania fastagiata* were included in the study as an outgroup using their internal transcribed spacer (ITS), external transcribed spacer (ETS) of nuclear ribosomal DNA (nrDNA) and Chloroplast *psbA-trnH* sequence data presented in GenBank NCBI (Appendix 2). These outgroup species were selected because their close systematic affinities to *Artemisia* as well as the availability of sequences in Genbank.

All the samples of *Artemisia* were obtained and collected on field trips in different locations of 5 districts of Gilgit-Baltistan region of Pakistan.

The voucher specimens collected from Pakistan were deposited in the herbarium of the Pakistan Museum of Natural History (PMNH). Further details of *Artemisia* samples employed for the analyses based on nrDNA (ITS, ETS) and cpDNA (*PsbA-trnH*) data, including taxonomic determination, voucher codes, and collection information can be found in Table 3.1.

3.7.1. DNA Extraction

After cleaning up the leaves with ethanol (70%), total genomic DNA was extracted primarily from the leaves by CTAB method given by Doyle and Doyle, (1990) and when needed, then Plant DNeasy kit (QIAGEN) was also used (Appendix 3).

3.7.2. CTAB Method

0.2 g of plant tissue was taken in a 2 ml eppendrof tube along with a grinding bead (3mm retsch cone ball) and was crushed by an electric mixer mill. Then preheated (at 65°C) 1 ml of 2% CTAB (Hexadecyltrimethylammonium bromide) and 2 $\mu\ell$ of β-mercaptoethanol were added along with a pinch of PVPP (PolyVinylPolyPyrrolidine). Mixture was incubated for 30 minutes at 65°C in the heated block. Samples were mixed twice during the incubation stage by inverting the tubes.

Tubes were removed from the heated block and were cooled to ambient temperature for 2-3 minutes. To each tube 500 $\mu\ell$ of chloroform-IAA (24:1) was added. After gently shaking and mixing, a momentary single phase was obtained.

Tubes were moved to the orbital shaker for 10-20 minutes shaking. Samples were then centrifuged for ten minutes at 13,000 rpm. The supernatant was recovered and moved to a clean 1.5 ml eppendrof tube and the chloroform extraction step was repeated.

The supernatant was removed to a clean 1.5 ml eppendrof tube. DNA was precipitated by adding 600 $\mu\ell$ of ice cold isopropanol and rocked gently.

Then tubes were centrifuged for 10 minutes at 13,000 remaining wash buffer to drain off. Pellet was dried in the vacuum centrifuge for 5 minutes.

Gently agitated to release the pellet from the lower part of the tube and left for at 30 minutes at room temperature.

The tubes were again centrifuged at 13,000 rpm for 5 minutes. Supernatant was removed and tubes were invert to allow the remaining wash buffer to drain off.

Pellet was dried in the vacuum centrifuge for 5 minutes. Pellet was dissolve in 50 to 100 µℓ of TE and mixed well and stored at -20 °C.

3.7.3. Quantification of Genomic DNA

Quantification of genomic DNA for each sample was estimated by measuring its purity A260/280 using an ND-2000 spectrometer (Urreizti *et al.*, 2012) (Nanodrop Technologies, Wilmington, DE, USA), and visual quality was assessed using agarose gel electrophoresis. The electrophoresis of extracted DNA was performed for 45 min at 100 voltages in 1% agarose gels, and subsequently visualized under the Trans illuminator UV light.

3.7.4. Dneasy Kit (QIAGEN) Method

This protocol was supplied by the manufacturers with DNeasy® kit (QIAGEN) in the form of a handbook (Appendix 3).

3.7.5. PCR conditions for genomic amplification

PCR amplifications were performed in 50 µℓ reaction volumes containing: 36 µℓ ddH_2O, 5 µℓ 1xPCR buffer, 2 µℓ deoxyribonucleoside triphosphates (dNTPs), 1 µℓ of MgCl2, 1.5 µℓ of forward and reverse primers for ITS (ITS9 and ITS6), ETS (ETS-AST1 and 18SETS) and chloroplast *psbA-trnH* (psbA3'f and trnHf) (Table 4). 1-1.5 µℓ of 20-50 ng of template DNA, 1 µℓ DMSO, 0.5 µℓ of 5 units *Taq polymerase* (Thermo Scientific, Maxima Hot Start) and 21 µℓ deionized water in an ABI thermo-cycle.

The PCR profile conducted for amplification of nuclear ITS9-6 region was as follow: pre-denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 1 minute or 55°C for 30 seconds, and extensions at 72°C for 1 minute, with final extension at 72°C for 5 minutes.

The PCR profile conducted for amplification of nuclear ETS region was as follow: pre-denaturation at 97°C for 2 min, followed by 36 cycles of denaturation at 97°C for 2 seconds, annealing at 55°C for 30 s, and extensions at 72°C for 30 s, with final extension at 72°C for 7 min.

The PCR conditions for chloroplast *psbA-trnH* were as follow: pre-denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 minute, extension at 72°C for 1.5 minute, with final extension at 72°C for 7 minutes. The electrophoresis of PCR products were carried out at 100 voltages for 45 min in a 1.5% agarose gel prepared in 1xTBE (Trisborate- ethylenediaminetetraacetic acid) buffer and consequently visualized under UV light.

The size of the amplified region was determined on the gel using 1kb DNA standard size markers (N-3232L, Biolabs Company) as shown in Appendix 4.

Table 3.4. The primer sequences used for amplifying ITS, ETS of nrDNA and *psbA-trnH* regions of cpDNA in *Artemisia* species.

Primer	**Sequence**	**Base length**	**Reference**
Forward primer for ITS	ITS9 (F): 5'-GGAAGGAGAAGTCGTAACAAGG-3'	22	Potter *et al.*, 2007
Reverse primer for ITS	ITS6 (R): 5'-TCCTCCGCTTATTGATATGC-3'	20	Potter *et al.*, 2007
Forward primer for ETS	AST-1(F): 5'-CGTAAAGGTGCATGAGTGGTGT-3'	22	Markos and Baldwin, 2001
Reverse primer for ETS	18S-ETS (R): 5'-ACTTACACATGCATGGCTTAATCT-3'	24	Baldwin and Markos, 1998
Forward primer for *psbA-trnH*	psbA3'f (F): 5'-GTTATGCATGAACGTAATGCTC-3'	22	Sang *et al.*, 1997
Reverse primer for *psbA-trnH*	trnHf (R): CGCGCATGGTGGATTCACAATCC-3'	23	Tate and Simpson, 2003

3.7.6. Gel Extraction Of PCR Product

This protocol was supplied by the manufacturers with QIAquick Gel Extraction Kit (QIAGEN) method (Appendix 5).

3.7.7. Nucleotide sequencing

The amplified DNA was sequenced from both strands, in the core UC Davis sequencing facility using ABI 3730 Capillary Electrophoresis Genetic Analyzers with ABI BigDye Terminator v3.1 Cycle Sequencing method. The primer set used for sequencing were ITS (ITS-9 F and ITS-6 R), ETS (AST-1 F and 18S-ETS R), and *psbA-trnH* (psbA3'f F and trnHf R) as shown in Table 3.4. Sequenced fragments were assembled using BioEdit and Sequencher 5.4.6 software (Gene codes Co.). The structure of nuclear ribosomal gene (ETS and ITS) used for the molecular phylogeny of *Artemisia* from Gilgit-Baltistan region of Pakistan is given in figure 3.3.

3.7.8. Sequence alignment

Sequences collected for three different markers, nrDNA-ETS (n=79), nrDNA-ITS (n=78), and cpDNA-*psbA-trnH* (n=65), of different *Artemisia* species collected from Gilgit-Baltistan region and those of obtained from GenBank were each aligned separately using MAFFT v7.272 (Katoh and Standley, 2013) (options: linsi) followed by manual adjustments. Two additional MSAs were generated by concatenating these three markers: one with maximum species coverage but with missing data (CAT79; n=79), and another with no missing data but with fewer taxa (CAT64). Thus, total of five separate MSAs were generated for phylogeny reconstructiton.

3.7.9. Model Selection and Phylogenetic Analysis

The best base substitution models were predated for the MSAs of each individual marker (ETS, ITS, and *psbA-trnH*). For CAT64, the best models were predicted for each partition representing different markers. For CAT79, the same model predicted for the marker-specific MSAs were used for phylogeny reconstruction. In all cases the best models were predicted using jModelTest v2.1.7 (Darriba *et al.*, 2012) (options: -f -g 4 -i -s 203 -S BEST -t ML). The best model was selected based on Bayesian information criterion (BIC).

The estimated model was then passed on to GARLI v2.0.1 (Zwickl, 2006) to generate a maximum likelihood tree. GARLI was executed under default conditions except for the following options (options: genthreshfortopoterm = 100000, significanttopochange = 0.00001, treerejectionthreshold = 50.0). Parameters values were estimated by GARLI. Four parallel searches were conducted in order to avoid selecting a tree lodged on a local optimum. Branches with length less than $1x10^{-8}$ substitution/site were collapsed. Bootstrap analysis was conducted with 1,000 replicates.

Artemisia ITS, ETS and *psbA-trnH* were first analyzed separately, using Bayesian inference to assess congruence among these nuclear markers. Then, the sequences from the three regions were aligned separately (ITS with 657 characters, ETS with 397 characters and *psbA-trnH* with 396 characters) and concatenated in a final aligned matrix of 1,450 characters. This separate and combined nrDNA and cpDNA dataset was analyzed using, maximum parsimony (MP), maximum likelihood (ML) and neighbor joining (NJ) to assess the circumscription of species within the genus *Artemisia*.

MrBayes v.3.2.1 software (Ronquist *et al.*, 2012) was used for BI analyses for ITS, ETS and *PsbA-trnH* substitution parameters estimated in different partitions for the pooled data. With four Metropolis Coupled Chains, two autonomous Markov Chain Monte Carlo (MCMC) analyses were run for 5,000000 generations, sampling every 100 groups (Malik *et al.*, 2017). The bestfitting DNA substitution model for BI analyses was nominated with Mr.Modeltest 2.3 (Nylander, 2004), GTR+I+G for the combined data set as well as for the individual cpDNA and nrDNA data sets was done. After the validation of average standard deviation of split frequencies to <0.0, the first 25% trees were discarded as 'burn in' and 1.0 potential scale reduction factor was approached for all factors. The samples left were merged to construct a 50% majority rule consensus trees for posterior probabilities.

The ML, MP and NJ analysis was performed with MEGA-7 and RAxML-HPC v.8 (Stamatakis, 2014), partitioning the combined nrDNA dataset in two different ITS, ETS and *psbA-trnH* regions as in the BI analysis. All the sequenced data for three markers (nrDNA ITS, nrDNA ETS and cpDNA *psbA-trnH*) of collected *Artemisia* species were deposited in the Genbank (https://www.ncbi.nlm.nih.gov/genbank/update/) under accession numbers as shown in Table 3.5, 3.6 and 3.7.

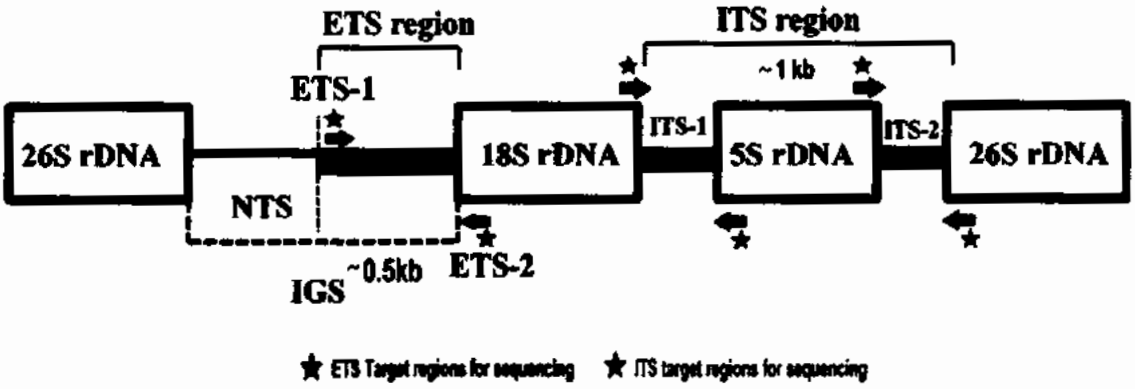

Figure 3.3. Structure of nuclear ribosomal gene (ETS and ITS) used for the molecular phylogeny of *Artemisia* from Gilgit-Baltistan region of Pakistan.

Table 3.5. Genbank reference of ITS nrDNA sequences of *Artemisia* species used in this study.

S/No	*Artemisia* Spp.	Genome	Submission No	GB Accession No	Date of submission
1	*A. annua*	nrDNA (ITS)	SUB3811644	MH091335	21-Mar-18
2	*A. argyi*	nrDNA (ITS)	SUB3811672	MH091340	21-Mar-18
3	*A. arborescens*	nrDNA (ITS)	SUB3845570	MH161334	04-Apr-18
4	*A. austriaca*	nrDNA (ITS)	SUB3821951	MH100692	22-Mar-18
5	*A. biennis*	nrDNA (ITS)	SUB3868783	MH161338	04-Apr-18
6	*A. campestris*	nrDNA (ITS)	SUB3816345	MH095575	21-Mar-18
7	*A. chamaemelifolia*	nrDNA (ITS)	SUB3822028	MH100697	22-Mar-18
8	*A. chinensis*	nrDNA (ITS)	SUB3821940	MH101881	22-Mar-18
9	*A. herba-alba*	nrDNA (ITS)	SUB3832678	MH113802	23-Mar-18
10	*A. indica*	nrDNA (ITS)	SUB3821713	MH100676	22-Mar-18
11	*A. maritima*	nrDNA (ITS)	SUB3868830	MH161339	04-Apr-18
12	*A. rutifolia*	nrDNA (ITS)	SUB3812028	MH092832	21-Mar-18
13	*A. scoparia*	nrDNA (ITS)	SUB3821931	MH100678	22-Mar-18
14	*A. sieberi*	nrDNA (ITS)	SUB3811875	MH091348	21-Mar-18
16	*A. tournefortiana*	nrDNA (ITS)	SUB3868828	MH161337	04-Apr-18
17	*A. verlotiorum*	nrDNA (ITS)	SUB3821701	MH100668	22-Mar-18
18	*A. vulgaris*	nrDNA (ITS)	SUB3832679	MH107243	23-Mar-18
19	*A.* sp. AD-H	nrDNA (ITS)	SUB3811865	MH094666	21-Mar-18
20	*A.* sp.-A	nrDNA (ITS)	SUB3816355	MH102419	22-Mar-18
21	*A.* sp.-B	nrDNA (ITS)	SUB3816371	MH104610	22-Mar-18
22	*A.* sp.-C	nrDNA (ITS)	SUB3821672	MH102417	22-Mar-18
23	*A.* sp.-D	nrDNA (ITS)	SUB3868848	MH168383	04-Apr-18
24	*A.* sp.-E	nrDNA (ITS)	SUB3822045	MH102420	22-Mar-18
25	*A.* sp.-F	nrDNA (ITS)	SUB3868834	MH168384	04-Apr-18
26	*A.* sp.-G	nrDNA (ITS)	SUB3821914	MH102418	22-Mar-18
27	*A.* sp.-H	nrDNA (ITS)	SUB3822068	MH102416	22-Mar-18
28	*A.* sp.-I	nrDNA (ITS)	SUB3812017	MH094656	21-Mar-18

Table 3.6. Genbank reference of ETS nrDNA sequences of *Artemisia* species used in this study.

S/No	*Artemisia* Spp.	Genome	Submission No	GB Accession No	Date of submission
1	*A. annua*	nrDNA (ETS)	BANKIT-2097930	MH257318	26-Apr-18
2	*A. argyi*	nrDNA (ETS)	BANKIT-2109228	MH257319	26-Apr-18
3	*A. arborescens*	nrDNA (ETS)	BANKIT-2111229	MH292877	03-May-18
4	*A. austriaca*	nrDNA (ETS)	BANKIT-2111231	MH292878	03-May-18
5	*A. biennis*	nrDNA (ETS)	BANKIT-2111249	MH292883	03-May-18
6	*A. campestris*	nrDNA (ETS)	BANKIT-2111191	MH292866	03-May-18
7	*A. chamaemelifolia*	nrDNA (ETS)	BANKIT-2111196	MH292867	03-May-18
8	*A. chinensis*	nrDNA (ETS)	BANKIT-2111225	MH292876	03-May-18
9	*A. gmelinii*	nrDNA (ETS)	BANKIT-2111233	MH292879	03-May-18
10	*A. herba-alba*	nrDNA (ETS)	BANKIT-2111244	MH292882	03-May-18
11	*A. indica*	nrDNA (ETS)	BANKIT-2111216	MH292873	03-May-18
12	*A. maritima*	nrDNA (ETS)	BANKIT-2111162	MH292863	03-May-18
13	*A. rutifolia*	nrDNA (ETS)	BANKIT-2111184	MH292865	03-May-18
14	*A. scoparia*	nrDNA (ETS)	BANKIT-2111222	MH292875	03-May-18
15	*A. sieberi*	nrDNA (ETS)	BANKIT-2111132	MH292862	03-May-18
17	*A. tournefortiana*	nrDNA (ETS)	BANKIT-2111199	MH292868	03-May-18
18	*A. verlotiorum*	nrDNA (ETS)	BANKIT-2111213	MH292872	03-May-18
19	*A. vulgaris*	nrDNA (ETS)	BANKIT-2111255	MH292876	03-May-18
20	*A.* sp. AD-H	nrDNA (ETS)	BANKIT-2109242	MH257320	26-Apr-18
21	*A.* sp.-A	nrDNA (ETS)	BANKIT-2111201	MH292869	03-May-18
22	*A.* sp.-B	nrDNA (ETS)	BANKIT-2111206	MH292870	03-May-18
23	*A.* sp.-C	nrDNA (ETS)	BANKIT-2111210	MH292871	03-May-18
24	*A.* sp.-D	nrDNA (ETS)	BANKIT-2111264	MH292886	03-May-18
25	*A.* sp.-E	nrDNA (ETS)	BANKIT-2111239	MH292880	03-May-18
26	*A.* sp.-F	nrDNA (ETS)	BANKIT-2111258	MH292885	03-May-18
27	*A.* sp.-G	nrDNA (ETS)	BANKIT-2111218	MH292874	03-May-18
28	*A.* sp.-H	nrDNA (ETS)	BANKIT-2111241	MH292881	03-May-18
28	*A.* sp.-I	nrDNA (ETS)	BANKIT-2111175	MH292864	03-May-18

Table 3.7. Genbank reference of *psbA-trnH* cpDNA sequences of *Artemisia* species used in this study.

S/No	*Artemisia* Spp.	Genome	Submission No	GB Accession No	Submission Date
1	*A. annua*	cpDNA (psba_trnh)	BANKIT-2112645	MH330156	08-May-18
2	*A. argyi*	cpDNA (psba_trnh)	BANKIT-2112789	MH330175	08-May-18
3	*A. arborescens*	cpDNA (psba_trnh)	BANKIT-2112666	MH330157	08-May-18
4	*A. austriaca*	cpDNA (psba_trnh)	BANKIT-2112754	MH330170	08-May-18
5	*A. biennis*	cpDNA (psba_trnh)	BANKIT-2112809	MH330179	08-May-18
6	*A. campestris*	cpDNA (psba_trnh)	BANKIT-2112723	MH330162	08-May-18
7	*A. chamaemelifolia*	cpDNA (psba_trnh)	BANKIT-2112816	MH330180	08-May-18
8	*A. chinensis*	cpDNA (psba_trnh)	BANKIT-2112753	MH330169	08-May-18
9	*A. gmelinii*	cpDNA (psba_trnh)	BANKIT-2112726	MH330163	08-May-18
10	*A. herba-alba*	cpDNA (psba_trnh)	BANKIT-2112773	MH330172	08-May-18
11	*A. indica*	cpDNA (psba_trnh)	BANKIT-2112742	MH330167	08-May-18
12	*A. maritima*	cpDNA (psba_trnh)	BANKIT-2112709	MH330160	08-May-18
13	*A. rutifolia*	cpDNA (psba_trnh)	BANKIT-2112716	MH330161	08-May-18
14	*A. scoparia*	cpDNA (psba_trnh)	BANKIT-2112747	MH330168	08-May-18
15	*A. sieberi*	cpDNA (psba_trnh)	BANKIT-2112705	MH330159	08-May-18
16	*A. tournefortiana*	cpDNA (psba_trnh)	BANKIT-2112778	MH330173	08-May-18
17	*A. verlotiorum*	cpDNA (psba_trnh)	BANKIT-2112737	MH330166	08-May-18
18	*A. vulgaris*	cpDNA (psba_trnh)	BANKIT-2112785	MH330174	08-May-18
19	*A.* sp. AD-H	cpDNA (psba_trnh)	BANKIT-2112697	MH330158	08-May-18
20	*A.* sp.-A	cpDNA (psba_trnh)	BANKIT-2112730	MH330164	08-May-18
21	*A.* sp.-B	cpDNA (psba_trnh)	BANKIT-2112823	MH330183	08-May-18
22	*A.* sp.-C	cpDNA (psba_trnh)	BANKIT-2112733	MH330165	08-May-18
23	*A.* sp.-D	cpDNA (psba_trnh)	BANKIT-2112817	MH330181	08-May-18
24	*A.* sp.-E	cpDNA (psba_trnh)	BANKIT-2112819	MH330182	08-May-18
25	*A.* sp.-F	cpDNA (psba_trnh)	BANKIT-2112796	MH330176	08-May-18
26	*A.* sp.-G	cpDNA (psba_trnh)	BANKIT-2112798	MH330177	08-May-18
27	*A.* sp.-H	cpDNA (psba_trnh)	BANKIT-2112804	MH330178	08-May-18
28	*A.* sp.-I	cpDNA (psba_trnh)	BANKIT-2112770	MH330171	08-May-18

CHAPTER 4

4. RESULTS

4.1. Ethnobotany

The list of *Artemisia* species is given in order by botanical name, with the data of their local name, distribution, parts used and life form. In this study, 15 *Artemisia* species with their potential folk and some common uses have been reported from Gilgit-Baltistan region of Pakistan. Along with some common *Artemisia* species such as, *A. annua*, *A. absinthium*, *A. sieversiana* and *A. maritima*, some rare species such as, *A. campestris*, *A. biennis*, *A. herba-alba*, *A. indica*, *A. vulgaris* and *A. scoparia* species have also been enlisted.

To the best of our knowledge, this study reported for the first time the ethnobotanical perspective and therapeutic potential of some rare *Artemisia* species like, *A. chamemelifolia*, *A. rutifolia*, *A. verlotiorum*, *A. austriaca and A. rutifolia* sub sp. from Gilgit-Baltistan region of Pakistan. The distribution of each species was also noted from the flora of Pakistan. The local names, part used along with their traditional usages is given below and also presented in Table 4.1.

1. *Artemisia absinthium* (Plate 4.1)

Local Name: Khakamus, Khakas, Kkayomon, Khalkhalush

Life form: Herb

Location: Gilgit, Ghizer, Astore, Hunza Nagar, Skardu and Chilaas

Part Used: Whole plant

Folk medicinal uses: Leaves are used to treat diabetes, vomiting, diarrhea and stomach worm. Its roots decoction is taken against piles. Leaf paste is used for joint pain. Leaf juice in small amount is given during pregnancy to ease labour pain. Leaves are used for flavor in local foods (Daodo, Soup). Leaf paste is used in antiaging remedies. Also used against fever, headache, back pain, dizziness and to treat insomnia.

Common uses: Used as insecticide. Utilized as a flavoring agent in food. Given to cow and other animals as fodder.

2. *Artemisia annua* (Plate 4.2)

Local Name: Kashuphaphring, Kakayomon, Khakas

Life form: Herb

Location: Gilgit, Astore and Ghizer

Part Used: Whole plant .

Folk medicinal uses: Leaves are used for the treatment of fever, cough, vomiting, diarrhea, stomach worm and chest pain. Diabetic people use its tea from dried leaves. Leaf paste is used to heal wounds. Decoction of whole plant is used to treat piles. Elderly people in Gilgit-Baltistan utilize it as an antiaging remedy.

Common uses: Live plant is used for ornamental purposes in Gilgit-Baltistan. Whole plant is used as insect repellent. It is also utilized as fodder for goat and sheep.

3. *Artemisia austriaca* (Plate 4.5)

Local Name: Nilo Zoon

Life form: Shrub

Location: Naltar, Bagrote Gilgit

Part Used: Leaves and Roots

Folk medicinal uses: Leaves are used to treat fever, pneumonia and chest related ailments. Roots are dipped in hair oil and used as antidandruff

Common uses: Used for ornamental purposes and fodder for goat.

4. *Artemisia biennis* (Plate 4.6)

Local Name: Bebari, Askor

Life form: Herb

Location: Gilgit, Skardu, Astore, Hunza Nagar, Ghizer and Chilaas

Part Used: Leaves, Flower and seeds

Folk medicinal uses: Leaves are used to relieve stomach pain and used against stomach worm. Leaves powder is also used to heal wounds.

Common uses: Seed powder is used as insect repellent

5. *Artemisia campestris* (Plate 4.7)

Local Name: Jaan, Jawn, Laheshi

Life form: Herb

Location: Gilgit and Skardu

Part Used: Whole plant

Folk medicinal uses: Leaves and flowers are used to treat cholera, fever, cough and pneumonia. Roots decoction is used to treat jaundice.

Common uses: Fodder for cow and donkey

6. *Artemisia chamaemelifolia* (Plate 4.8)

Local Name: Jhao, Kakayomon

Life form: Herb

Location: Naltar Gilgit, Ghizer, Hunza Nagar and Skardu

Part Used: Whole plant

Folk medicinal uses: Leaves decoction is given to children as an anthelmintic. Also used to stop loose motions.

Common uses: Fodder for goat and sheep

7. *Artemisia herba-alba* (Plate 4.14)

Local Name: Charah, Sharajay, Jhawo

Life form: Shrub

Location: Gilgit, Ghizer, Hunza Nagar, Astore, Skardu and Chilas

Part Used: Whole plant

Folk medicinal uses: Leaves are used to control high blood pressure, to treat gastric problems, diarrhoea and abdominal cramping. Leaf juice is also ingested to treat diabetes. Also used for urinary problems. Roots decoction is used to treat jaundice.

Common uses: Used as a source of fuel and employed for shelter purpose

8. *Artemisia indica* (Plate 4.15)

Local Name: Khakas

Life form: Herb

Location: Astore, Ghizer and Skardu

Part Used: Leaves and roots

Folk medicinal uses: Leaf decoction is used against diarrhoea, abdominal cramping and to kill stomach worm. Leaf paste is used for skin diseases. Root extract is used to relieve the kidney pain.

Common uses: Whole plant is used as insecticidal and mosquito repellent

9. *Artemisia maritima* (Plate 4.16)

Local Name: Zoon, Kino Zoon, Biralis, Bhursay

Life form: Shrub

Location: Gilgit, Skardu, Ghizer, Hunza Nagar and Astore

Part Used: Whole plant

Folk medicinal uses: The leaves are used as stomachic, fever, diarrhoea and to normalize high blood pressure. Leaf decoction is used against malaria and mosquito bite. The plant is given to children for stomach-ache and to kill the stomach worm. Its roots decoction is used as a cure for jaundice. Diabetic people also use its dried leaves tea.

Common uses: Insect repellent and fodder for goat and sheep. Used for fuel and shelter purpose

10. *Artemisia rutifolia* (Plate 4.19)

Local Name: Brom-mon, Zoon, Karbhursay

Life form: Shrub

Location: Naltar Valley Gilgit

Part Used: Flower

Folk medicinal uses: The dried flowers are grinded and its powdered and used as anthelmintic especially for children.

Common uses: Used as a source of fuel for villagers.

11. *Artemisia-rutifolia* sub sp (Plate 4.20)

Local Name: Shao Zoon, Karbhursay, Karphobursay

Life form: Shrub

Location: Naltar, Gilgit, Hunza Nagar

Part Used: Whole Plant

Folk medicinal uses: Decoction of leaves is used for coughs, and fever. The tea prepared from dried leaves is given to women during child birth to relieve pain

Common uses: Used for ornamental purpose and to make roofs in villages of Gilgit Baltistan. Also used for fuel purposes

12. *Artemisia scoparia* (Plate 4.21)

Local Name: Khasmer phyahma, Jhao

Life form: Herb

Location: Gilgit, Skardu and Astore

Part Used: Whole plant

Folk medicinal uses: Whole plant is used as diuretic, purgative, antiinflamation and for the treatment of jaundice. Leaves are employed as an insecticidal

Common uses: Used for fuel purpose and given as fodder for animals

13. *Artemisia sieversiana* (Plate 4.23)

Local Name: khakhamus, Khakas

Life form: Herb

Location: Gilgit, Ghizer, Hunza, Skardu Baltistan

Part Used: Leaves stem and Roots

Folk medicinal uses: The leaves and stems are used against stomach worm and to treat vomiting. A decoction of the plant is used to relieve joints pain. Roots extract is applied to swellings. Leaves decoction is used with local foods as flavouring and antiaging remedy.

Common uses: Fodder for cow and goat

14. *Artemisia verlotiorum* (Plate 4.25)

Local Name: Khakhalus

Life form: Herb

Location: Gahkuch, Sherqila, Bubar Ghizer

Part Used: leaves and stem

Folk medicinal uses: Leaves are used against stomach related problems and stem is used as miswak to brush teeth.

Common uses: Fodder for goat

15. *Artemisia vulgaris* (Plate 4.26)

Local Name: Phamering

Life form: Herb

Location: Khunjerab, Gojal Hunza Nagar, Gilgit and Skardu

Part Used: Leaves and stem

Folk medicinal uses: Leaves decoction is used to treat fever and cough. Also used as antiinflamation and wound healer.

Common uses: Fodder for goat and sheep

Among the 15 studied *Artemisia* species, classification of the plants on the basis of their habit showed 10 herbs and 5 shrubs (Figure 4.1). On the basis of their life cycle, 1 species was annual, 2 were biannual and the remaing 12 were perennial (Figure 4.2). Regarding the utilization of plant parts, 8 *Artemisia* species are used as whole and only some parts of the remaining 7 plants are used as shown in figure 4.3. Most of the *Artemisia* species were used for numerous ailments. Overall, more than 30 different health-related applications were reported. 5 species are used in diarrhoea and 3 in vomiting, 6 species are used in fever, 4 species are utilized for cough, colds, flu, pneumonia and chest pain. 8 species are utilized against stomach cramping and worms, 8 plants species in pain relief, wound healing and swellings. 4 species are used for diabetes and blood pressure, 2 species are used to get rid of piles, 1 species is used for urinary concerns. 2 for the treatment of acidity and gastric problems, 4 species are used for jaundice and cholera, 1 species is used for dental concerns, 2 species are utilized for malaria and mosquito bite, 1 species is for insomnia and dizziness, 3 species are employed for antiinflamation and 1 for skin infections, 6 species are utilized as an ant insecticidal, 3 species are taken as an antiaging remedy flavouring agent in local foods. Typically, various parts of *Artemisia* plants are prepared/consumed with water, tea, sugar and milk. Common preparations include powder and decoction etc.

Table 4.1. Folk medicinal uses of *Artemisia* species from Gilgit-Baltistan region of Pakistan

S/NO	*Artemisia* spp.	Local Name	Life form	Local Distribution	Part used	Folk medicinal uses	Common uses
1.	*A. absinthium*	Khakamus, Khakas, Kkayomon, Khalkhalush	Herb	Gilgit,Ghizer, Astore, Hunza Nagar, Skardu and Chilaas	Whole plant	Leaves are used to treat diabetes, vomiting, diarrhea and stomach worm. Decoction of roots is also used against piles. Leaf paste is used against joint pain. Leaf juice in small amount is given during pregnancy to women to ease labour pain. Leaves paste is used as flavour enhancer in local foods (Daodo, Soup) and taken as antiaging remedy. Also used against fever, headache, back pain, dizziness and to treat insomnia	Used as insecticide. Used as flavoring agent in foods. Used as fodder for cow and other animals
2.	*A. annua*	Kashuphaphring, Kakayomon, Khakas	Herb	Gilgit, Astore and Ghizer	Whole plant	Leaves are used to treat fever, cough, vomiting, diarrhea, stomach worm and chest pain. Diabetic patients use dried leaves for making green tea. Leaf paste is used to heal wounds. Decoction of whole plant is used to treat piles. Aged people in Gilgit-Baltistan utilize it as an antiaging remedy	Live plant is used for ornamental purposes in Gilgit-Baltistan. Whole plant is used as insect repellent. It is also utilized as fodder for goat and sheep
3.	*A. austriaca*	Nilo Zoon	Shrub	Naltar, Bagrote Gilgit	Leaves and roots	Leaves are used to treat fever, pneumonia and chest related ailments. Roots are dipped in hair oil and used as antidandruff	Used for ornamental purposes and used as fodder for goat
4.	*A. biennis*	Bebari, Askor	Herb	Gilgit, Skardu, Astore, Hunza Nagar, Ghizer and Chilaas	Leaves flower and seeds	Leaves are used to relieve stomach pain. Used against stomach worm and to heal wounds	Seed powder is used as insect repellent
5.	*A. campestris*	Jaan, Jawn, Laheshi	Herb	Gilgit and Skardu	Whole plant	Leaves and flowers are used to treat cholera, fever, cough and pneumonia. Roots are used to treat jaundice	Fodder for cow and donkey

Table 4.1. Continued....

S/NO	*Artemisia* spp.	Local Name	Life form	Local Distribution	Part used	Folk medicinal uses	Common uses
6.	*A. chamemelifolia*	Jhao, Kakayomon	Herb	Naltar Gilgit, Ghizer, Hunza Nagar and Skardu	Whole plant	Leaves decoction is given to children as an athhelmintic. Also used to stop loose motions	Fodder for goat and sheep
7.	*A. herba-alba*	Charah, Sharajay, Jhawo	Shrub	Gilgit, Ghizer, Hunza Nagar, Astore, Skardu and Chilas	Whole plant	Leaves are used to control high blood pressure, to treat gastric problems, diarrhea and abdominal cramping. Leaf juice is also ingested to treat diabetes. Also used for urinary problems Roots are used to treat jaundice	Used as a source of fuel and employed for shelter purpose
8.	*A. indica*	Khakas	Herb	Astore, Ghizer and Skardu	Leaves and roots	Leaf decoction is used for diarrhea and abdominal cramping and stomach worm. Leaf paste is used for skin diseases. Root extract is used to relieve pain, especially kidney pain	Whole plant is used as insecticidal and mosquito repellent
9.	*A. maritima*	Zoon, Kino Zoon, Biralis, Bhursay	Shrub	Gilgit, Skardu, Ghizer, Hunza Nagar and Astore	Whole plant	The leaves are used as stomachic, fever and diarrhea and to normalize high blood pressure. Leaf decoction is used against malaria and mosquito bite. The plant is given to children for stomach-ache and to kill the stomach worm. Its roots are used as a cure for jaundice. Diabetic people also use its dried leaves tea	Used as Insect repellent and fodder for goat and sheep. Used for fuel and shelter purpose
10.	*A. rutifolia*	Brom-mon, Zoon, Karbhursay	Shrub	Naltar Valley Gilgit	Flower	The dried flowers are grinded and powder is used as anthelmintic especially for children	Used as a source of fuel for villagers

Table 4.1. Continued....

S/NO	*Artemisia* spp.	Local Name	Life form	Local Distribution	Part used	Folk medicinal uses	Common uses
11.	*A. rutifolia* sub sp.	Shao Zoon, Karbhursay, Karphobursay	Shrub	Naltar, Gilgit, Hunza Nagar	Whole plant	Decoction of leaves is used for coughs, and fever. The tea prepared from dried leaves is given to women during child birth to relieve pain	Used for ornamental purpose and to make roofs in villages of Gilgit-Baltistan. Also used for fuel purposes
12.	*A. scoparia*	Khasmer phyahma, Jhao	Herb	Gilgit, Skardu and Astore	Whole plant	Whole plant is used as diuretic, purgative, antiinflamation and for the treatment of jaundice. Leaves are employed as an insecticidal	Used for fuel purpose and given as fodder for animals
13.	*A. sieversiana*	khakhamus, Khakas	Herb	Gilgit, Ghizer, Hunza, Skardu Baltistan	Leaves stem and roots	Leaves and stem are used against stomach worm and vomiting. A decoction of the plant is used to relieve joints pain. Roots extract is applied to swellings. Used with local foods as flavoring and antiaging remedy	Fodder for cow and goat
14.	*A. verlotiorum*	Khakhalus	Herb	Gahkuch, Sherqila, Bubar Ghizer	Leaves and stem	Leaves are used against stomach related problems and stem is used as miswak to brush teeth	Fodder for goat
15.	*A. vulgaris*	Phamering	Herb	Khunjerab, Gojal Hunza Nagar, Gilgit and Skardu	Leaves and stem	Leaves decoction is used to treat fever and cough. Also used as antiinflamation and wound healer	Fodder for goat and sheep

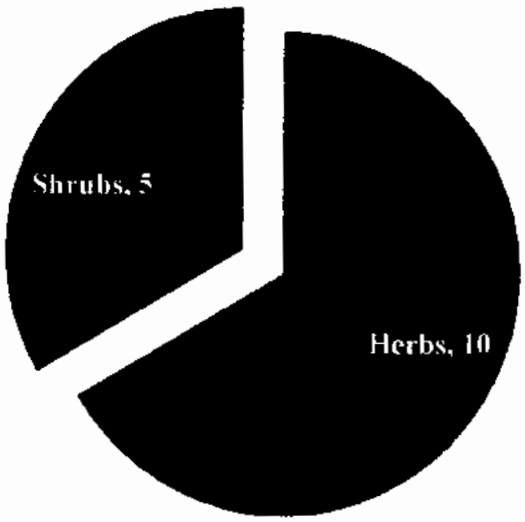

Figure 4.1. Distribution of *Artemisia* species for ethnobotanical investigation on the basis of habit.

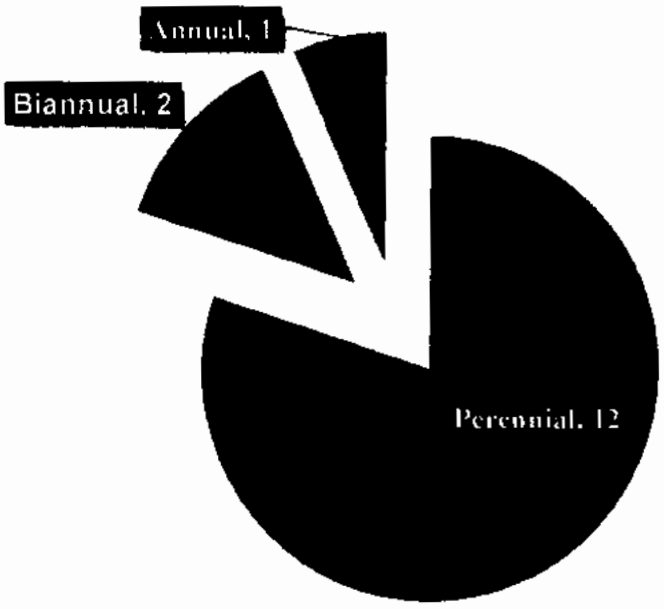

Figure 4.2. Distribution of *Artemisia* species for ethnobotanical investigation on the basis of life cycle.

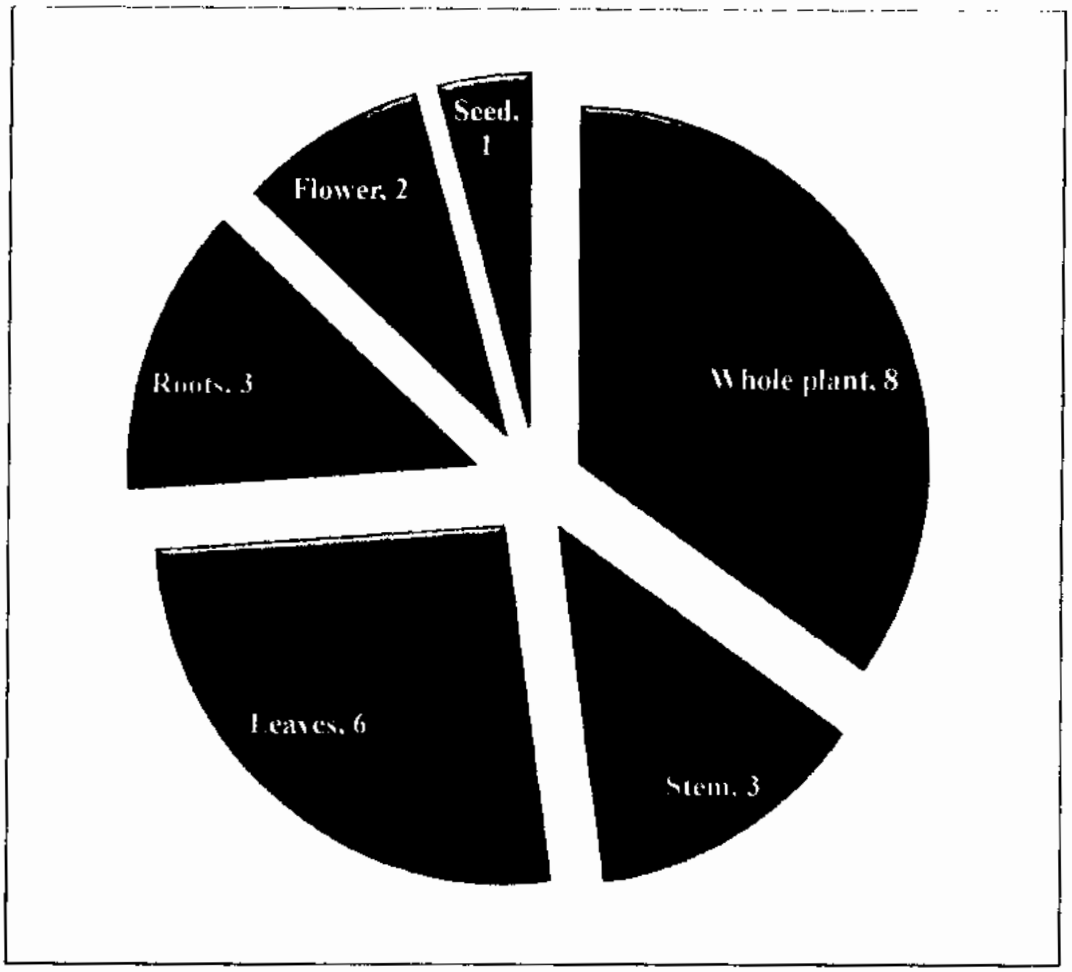

Figure 4.3. Distribution of ethnobotanicaly used parts of *Artemisia* plants.

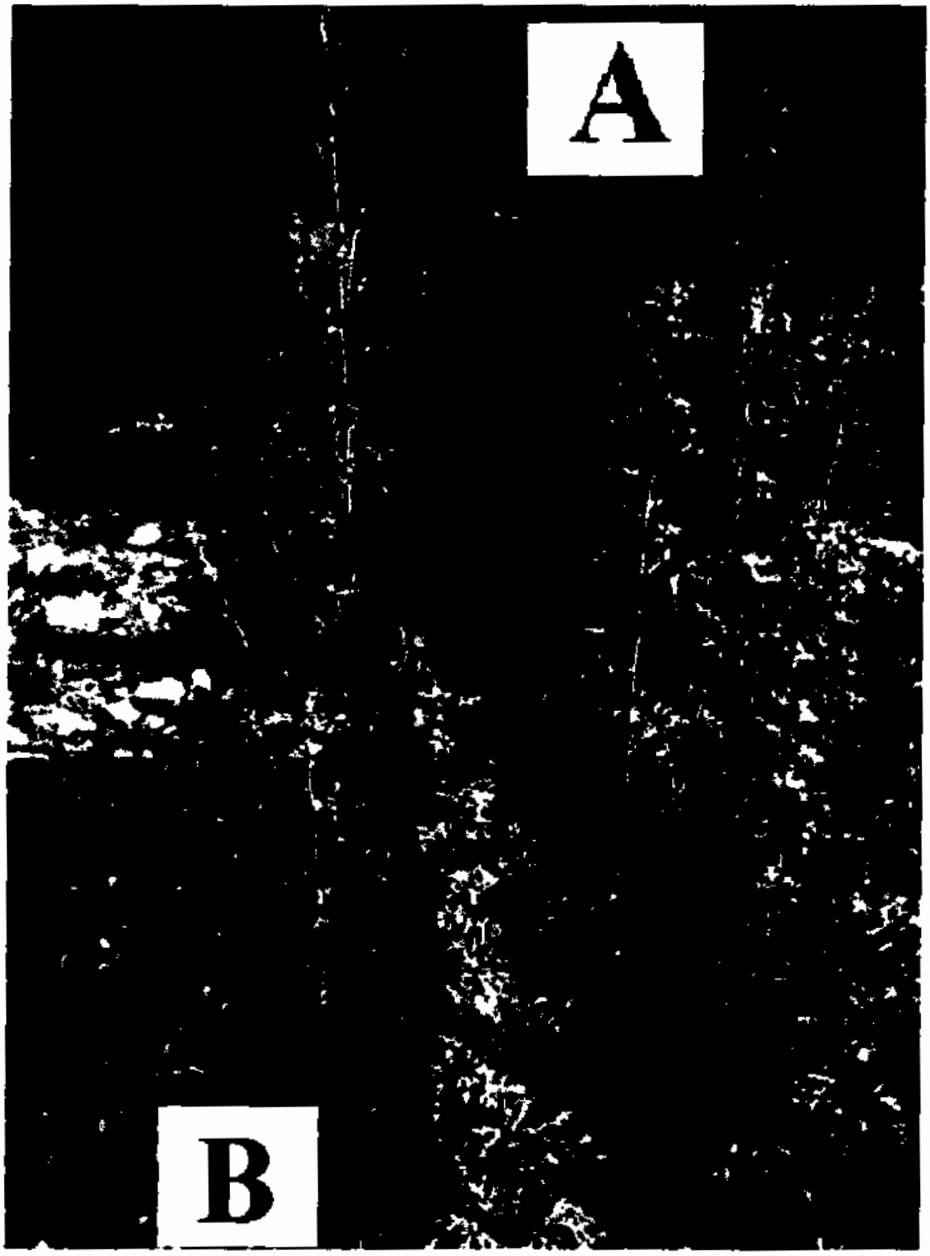

Plate 4.1. *Artemisia absinthium*

A= Plant; B= Florescence

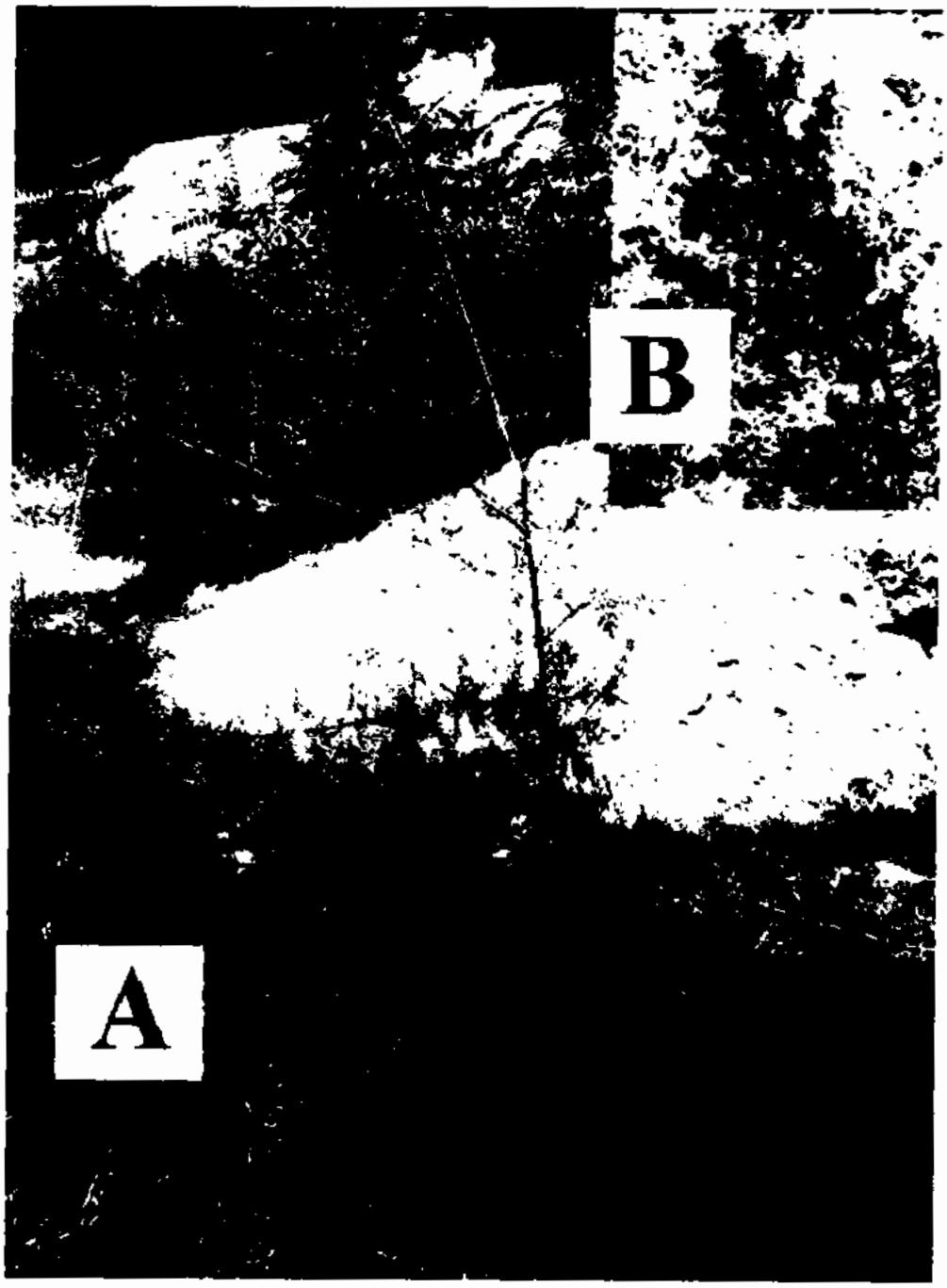

Plate 4.2. *Artemisia annua*

A= Plant; B= Florescence

Plate 4.3. *Artemisia arborescens*

Plate 4.4. *Artemisia argyi*

Plate 4.5. *Artemisia austriaca*

Plate 4.6. *Artemisia biennis*

A= Plant; B= Florescence

Plate 4.7. *Artemisia campestris*

A= Plant; B= Florescence

Plate 4.8. *Artemisia chamaemelifolia*

A= Plant; B= Florescence

Plate 4.9. *Artemisia chinensis*

A= Plant; B= Florescence

Plate 4.10. *Artemisia capillaris*

Plate 4.11. *Artemisia dubia*

Plate 4.12. *Artemisia sp.* AD-H

A= Plant; B= Leaves; C= Florescence

Plate 4.13. *Artemisia gmelinii*

A= Plant; B= Florescence

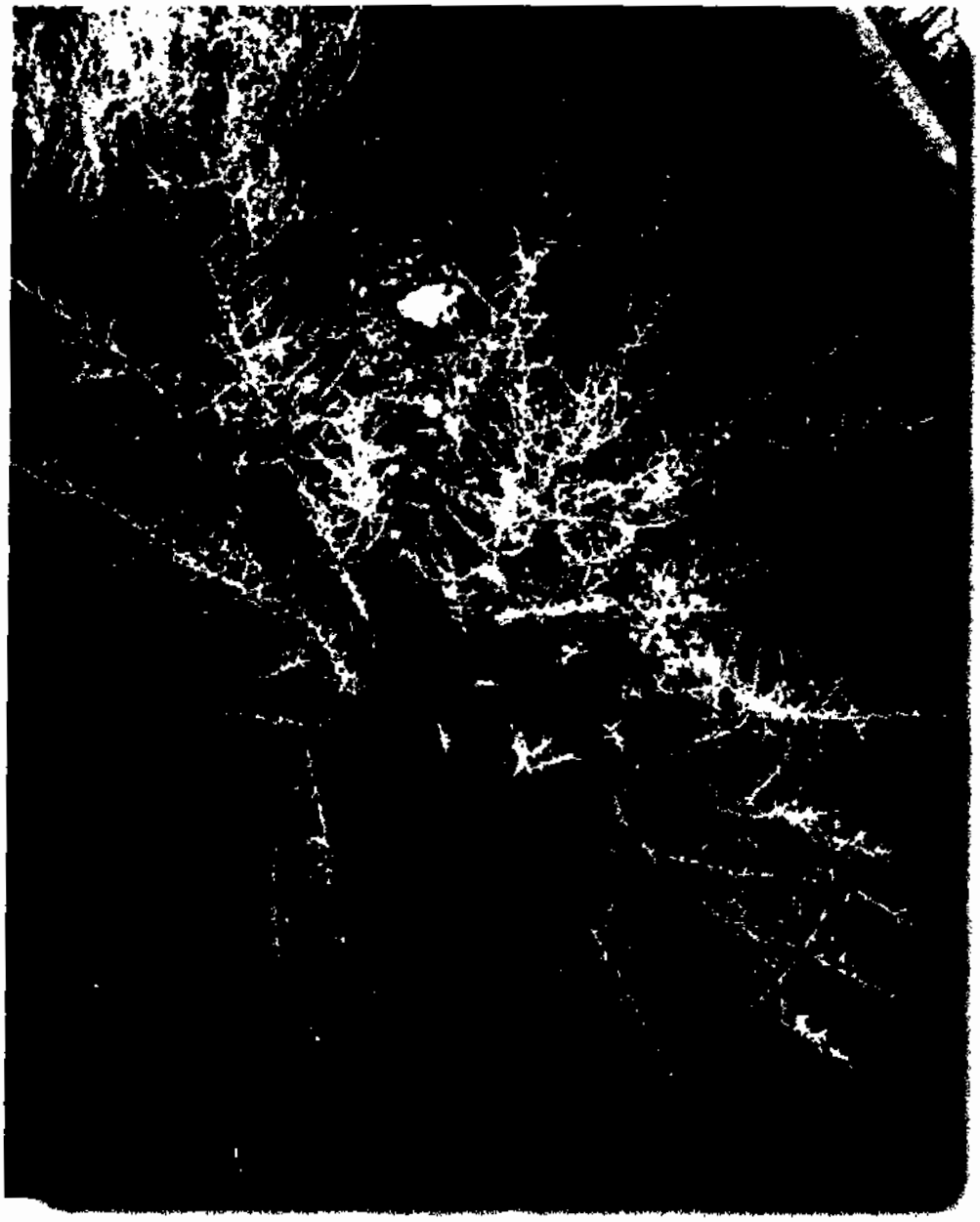

Plate 4.14. *Artemisia herba-alba*

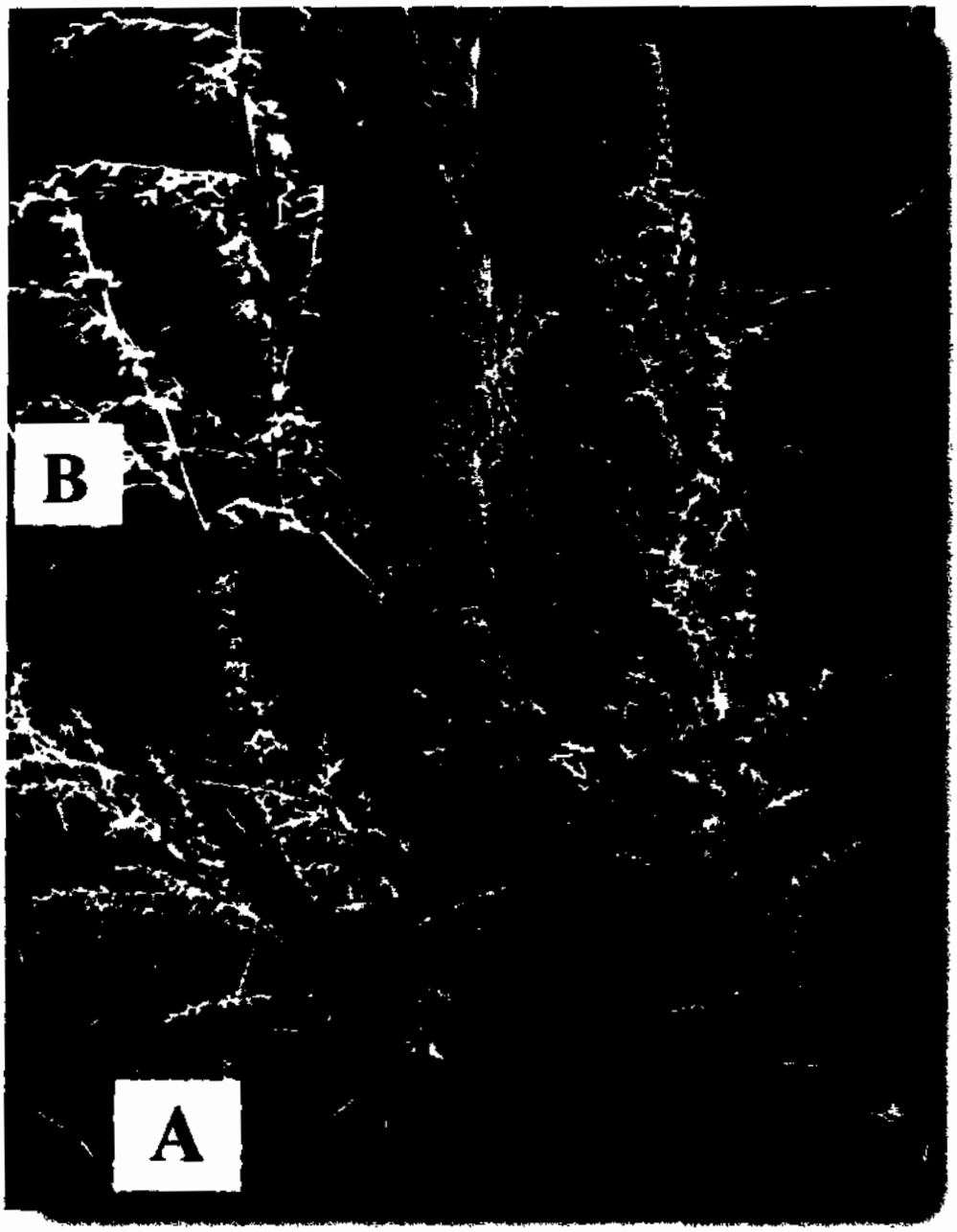

Plate 4.15. *Artemisia indica*

A= Plant; B= Florescence

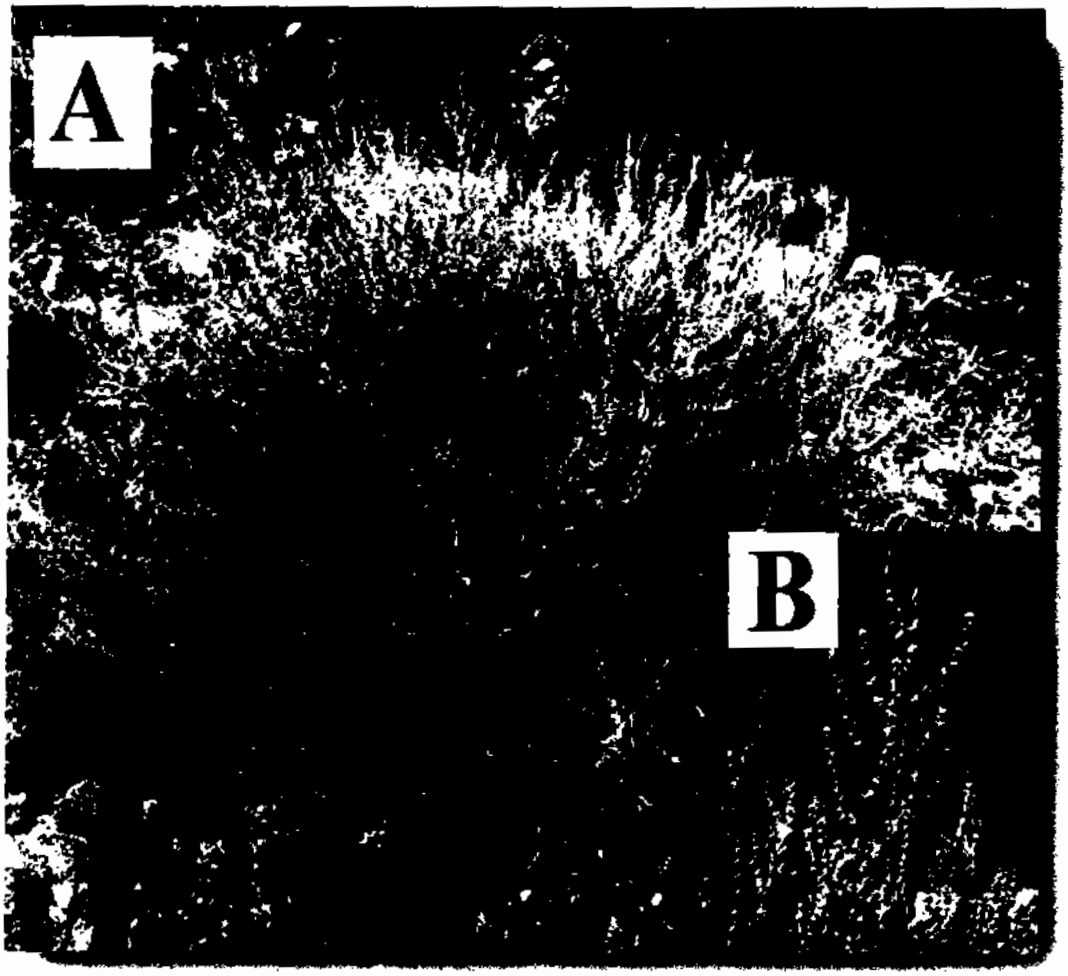

Plate 4.16. *Artemisia maritima*

A= Plant; B= Florescence

Plate 4.17. *Artemisia montana*

A= Plant; B= Florescence

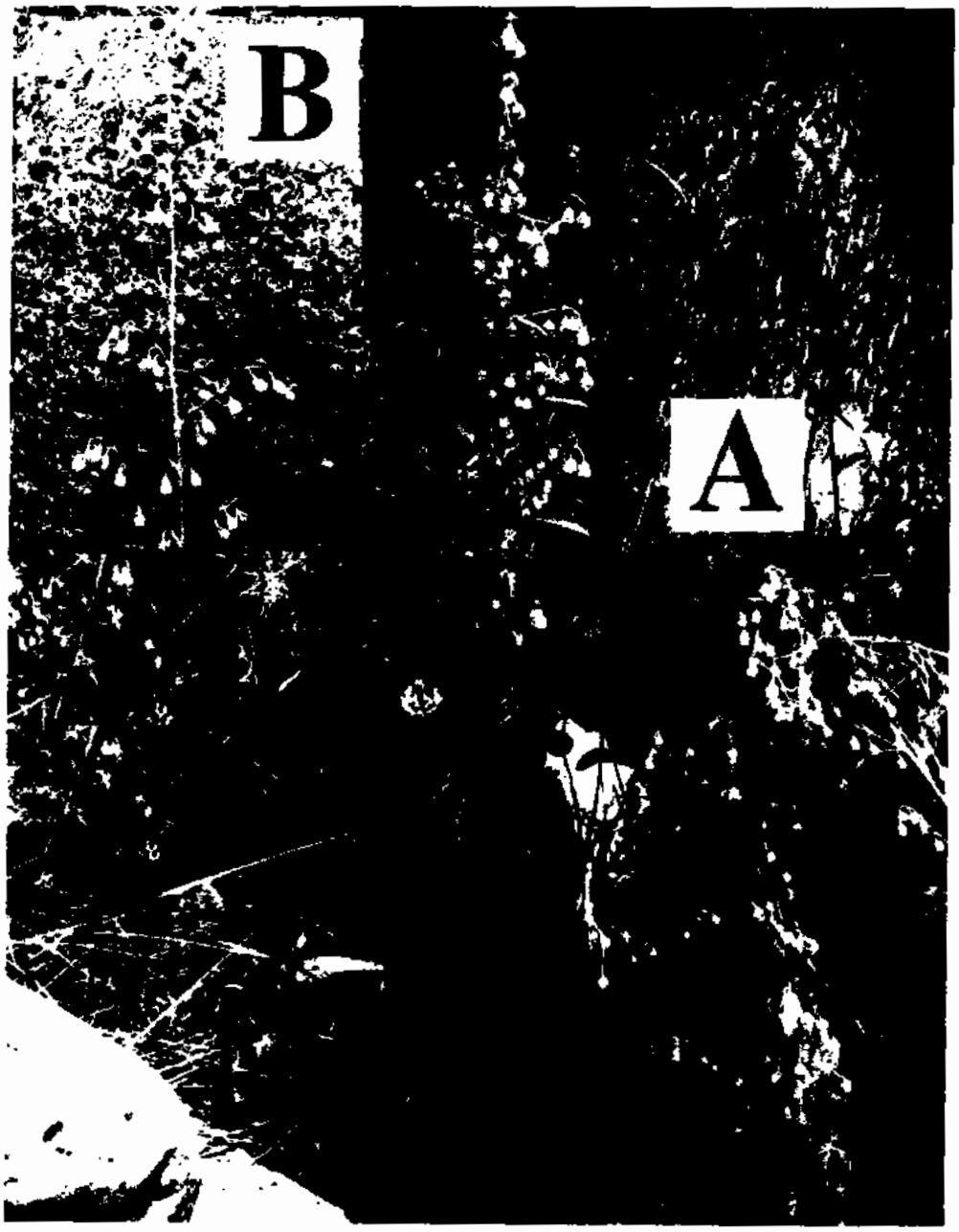

Plate 4.18. *Artemisia pontica*

A= Plant; B= Florescence

Plate 4.19. *Artemisia rutifolia*

A= Plant; B= Florescence

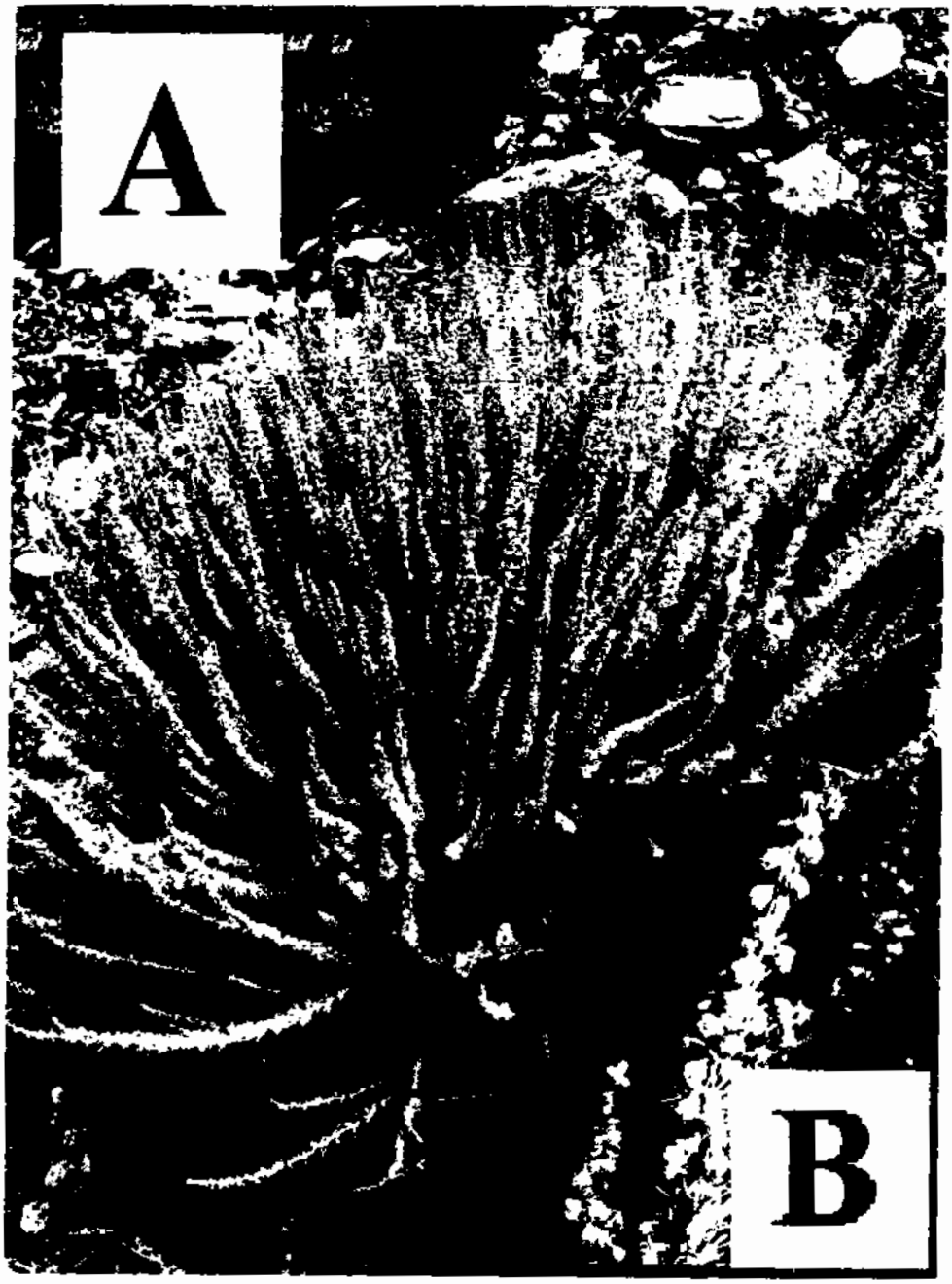

Plate 4.20. ***Artemisia rutifolia*** **sub sp.**

A= Plant; B= Florescence

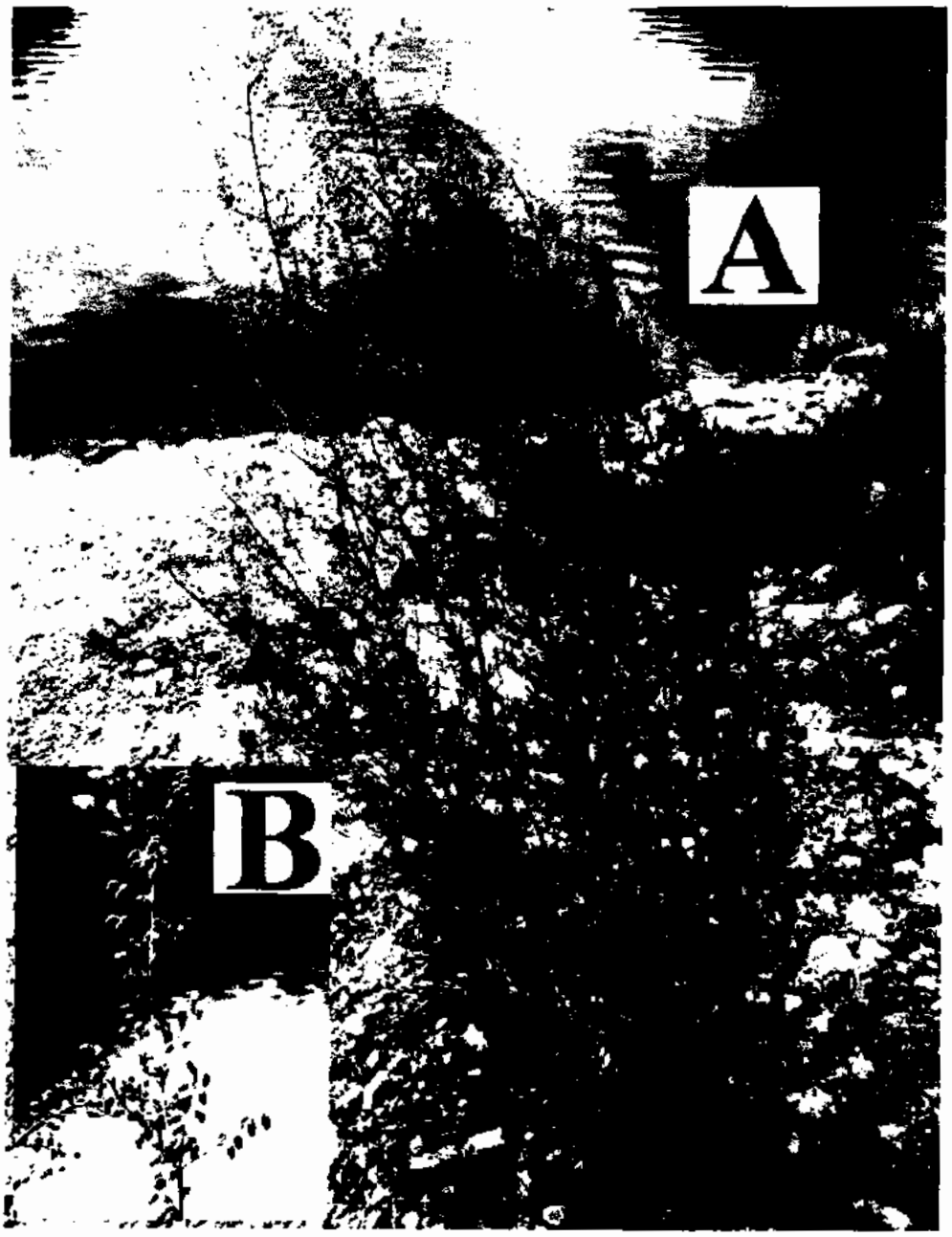

Plate 4.21. *Artemisia scoparia*

A= Plant; B= Florescence

Plate 4.22. *Artemisia sieberi*

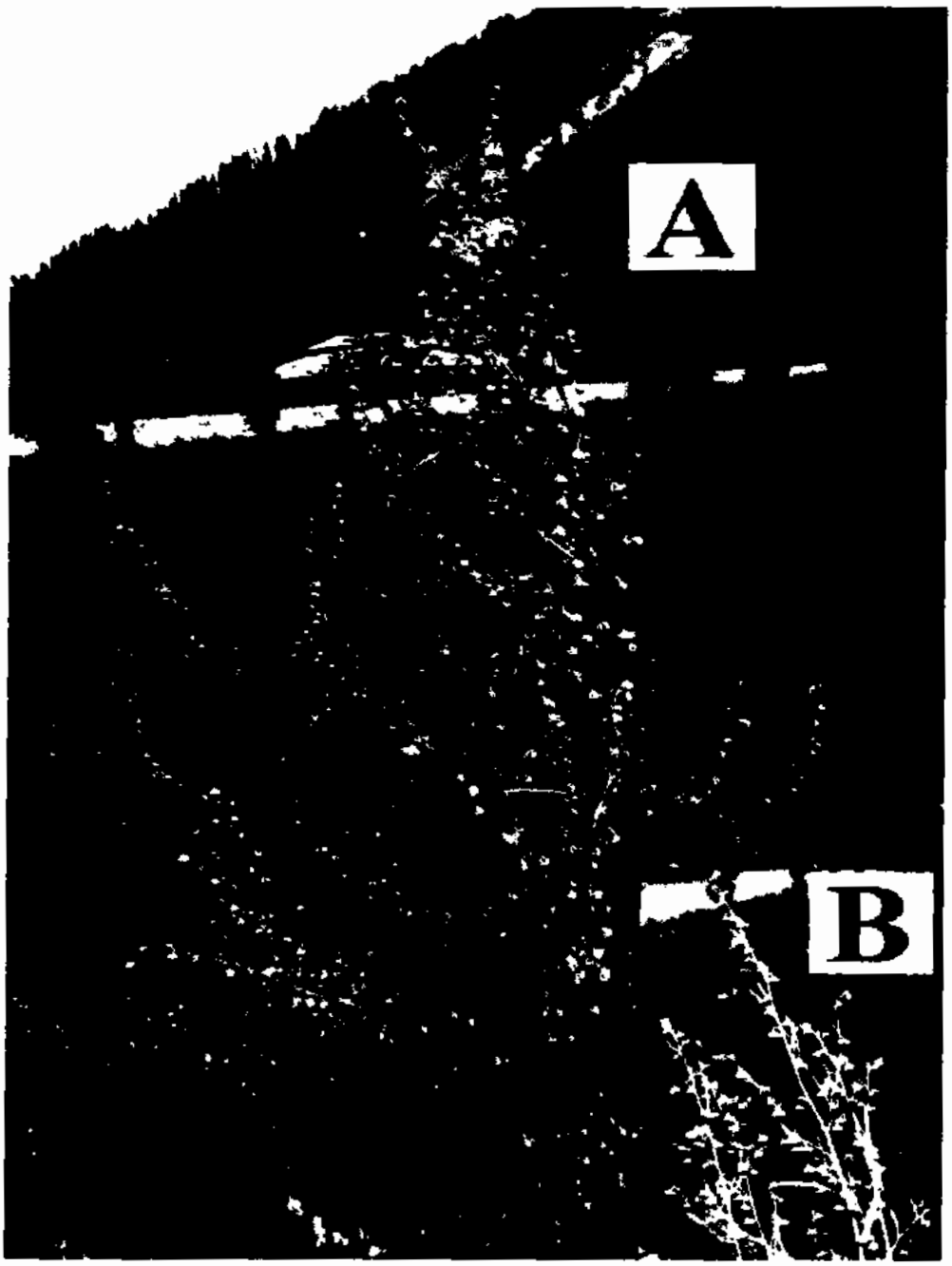

Plate 4.23. ***Artemisia sieversiana***

A= Plant; B= Florescence

Plate 4.24. *Artemisia tournefortiana*

A= Plant; B= Florescence

Plate 4.25. ***Artemisia verlotiorum***

A= Plant; B= Florescence

Plate 4.26. *Artemisia vulgaris*

A= Plant; B= Florescence

Plate 4.27. *Artemisia* sp. -A

A= Plant; B= Florescence

Plate 4.28. *Artemisia* sp. -B

A= Plant; B= Florescence

Plate 4.29. *Artemisia* sp. -C

A= Plant; B= Florescence

Plate 4.30. *Artemisia* sp. -D

A= Plant; B= Florescence

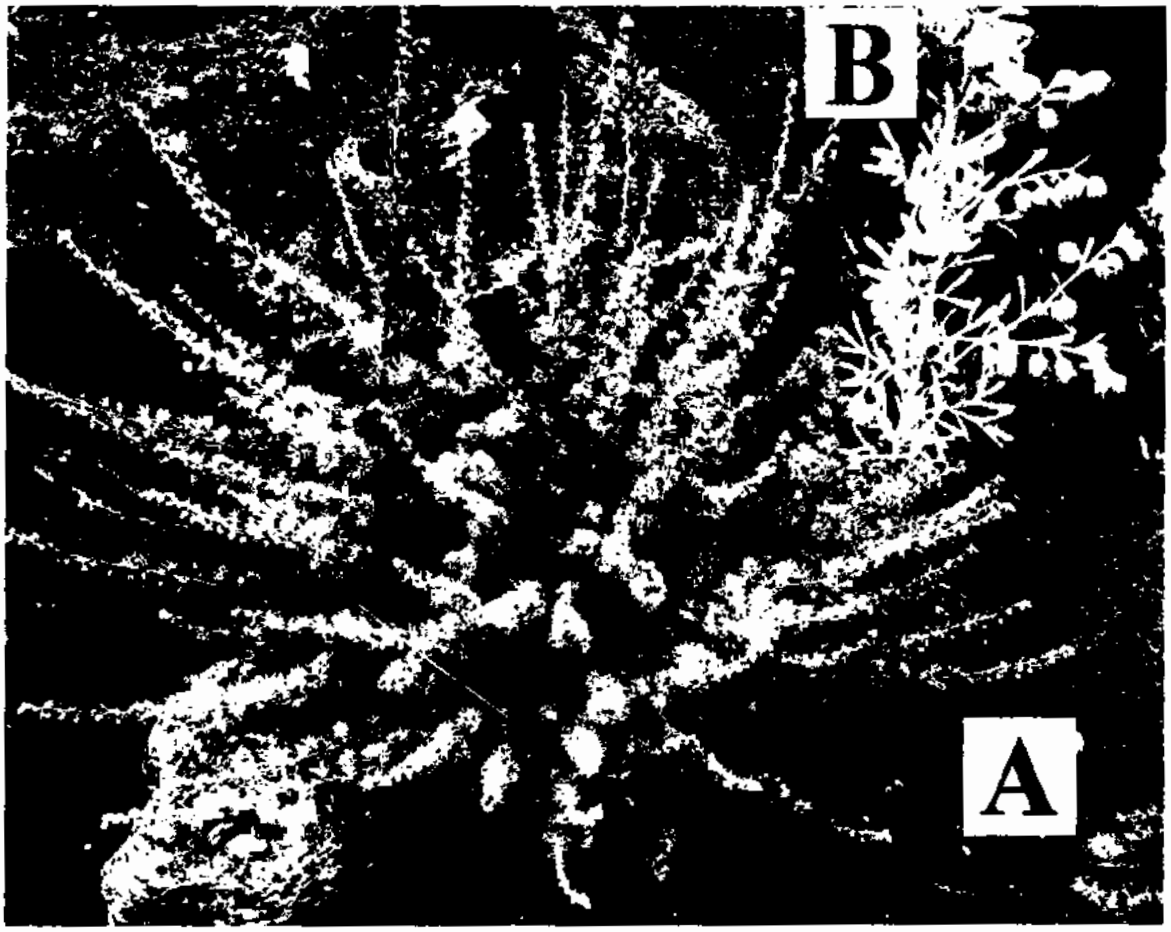

Plate 4.31. *Artemisia* sp. -E

A= Plant; B= Florescence

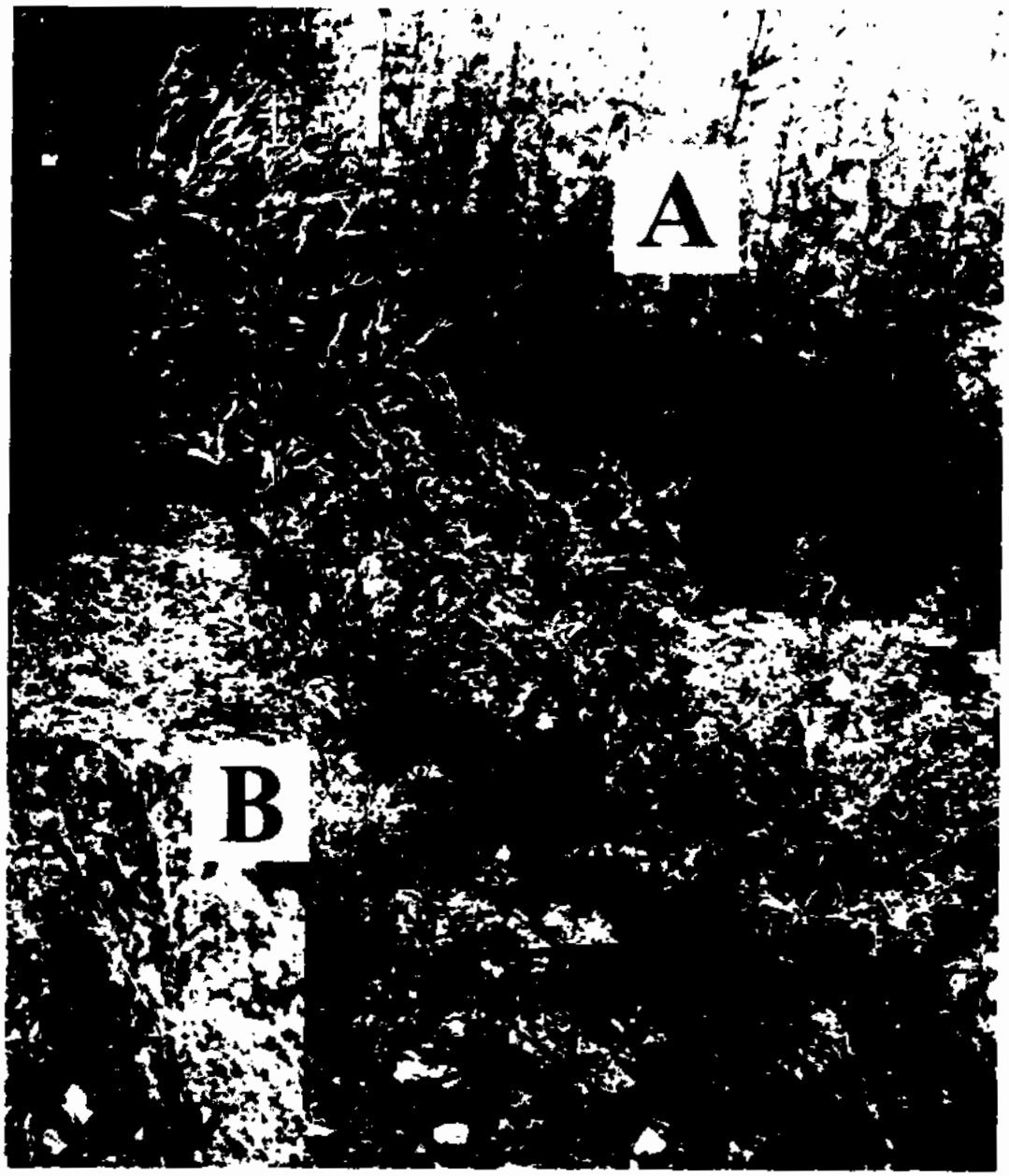

Plate 4.32. *Artemisia* sp. -F

A= Plant; B= Florescence

Plate 4.33. *Artemisia* sp. -G

A= Plant; B= Florescence

Plate 4.34. *Artemisia* sp. -H

A= Plant; B= Florescence

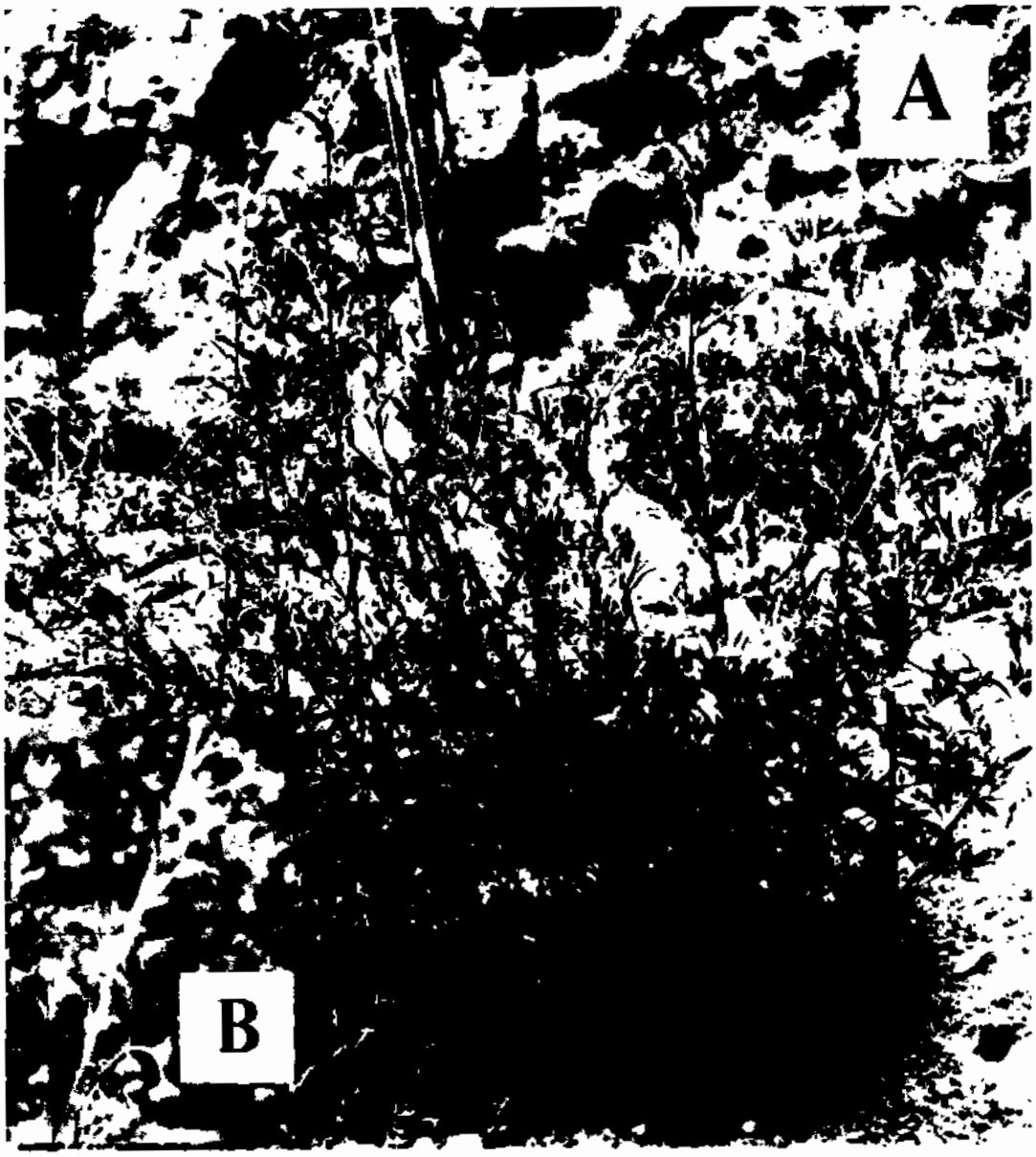

Plate 4.35. *Artemisia* sp. -I

A= Plant; B= Florescence

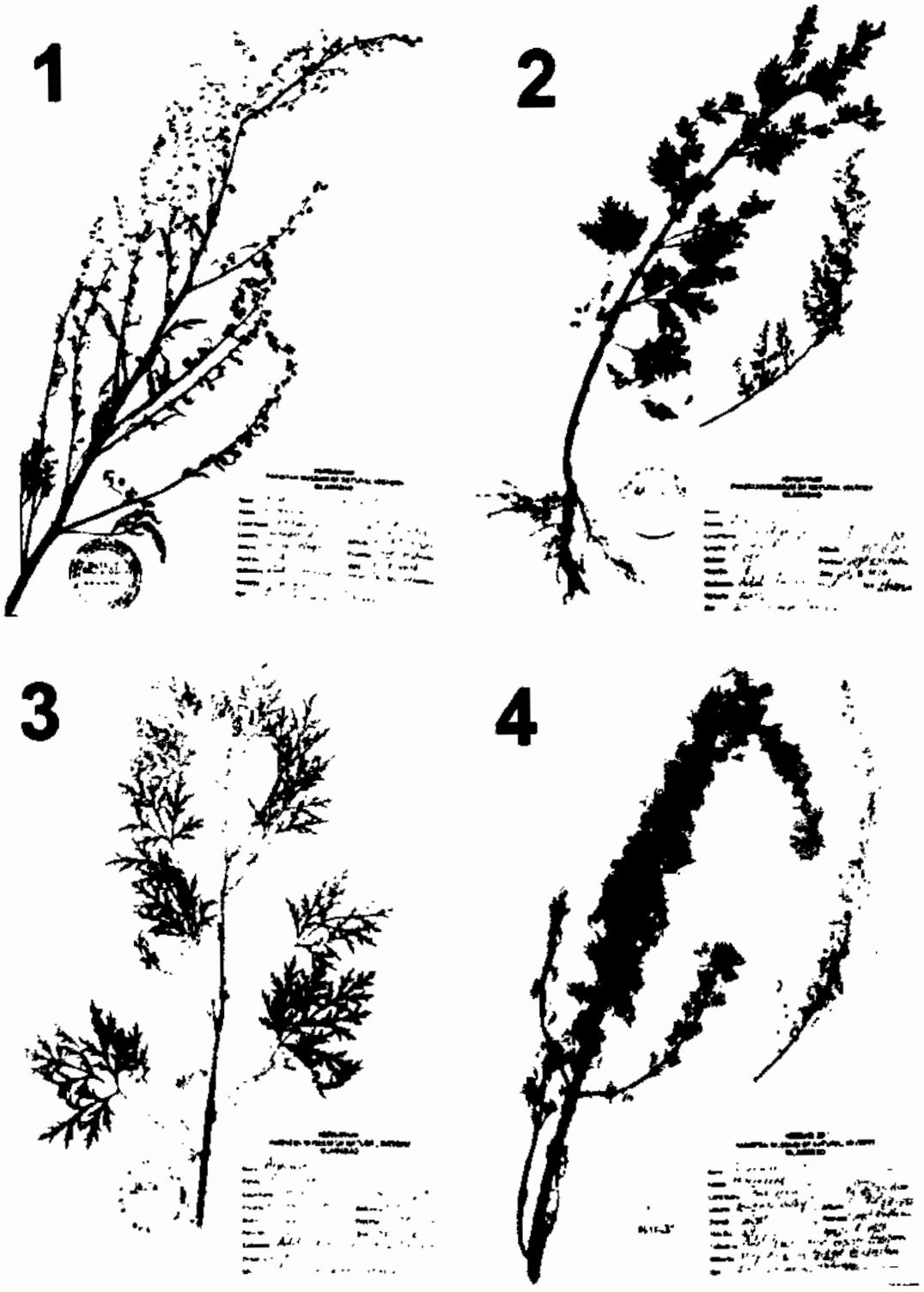

Plate 4.36. Voucher specimen of studied *Artemisia* species. 1= *A. absinthium*; 2= *A. annua*; 3= *A. argyi*; 4= *A. austriaca*

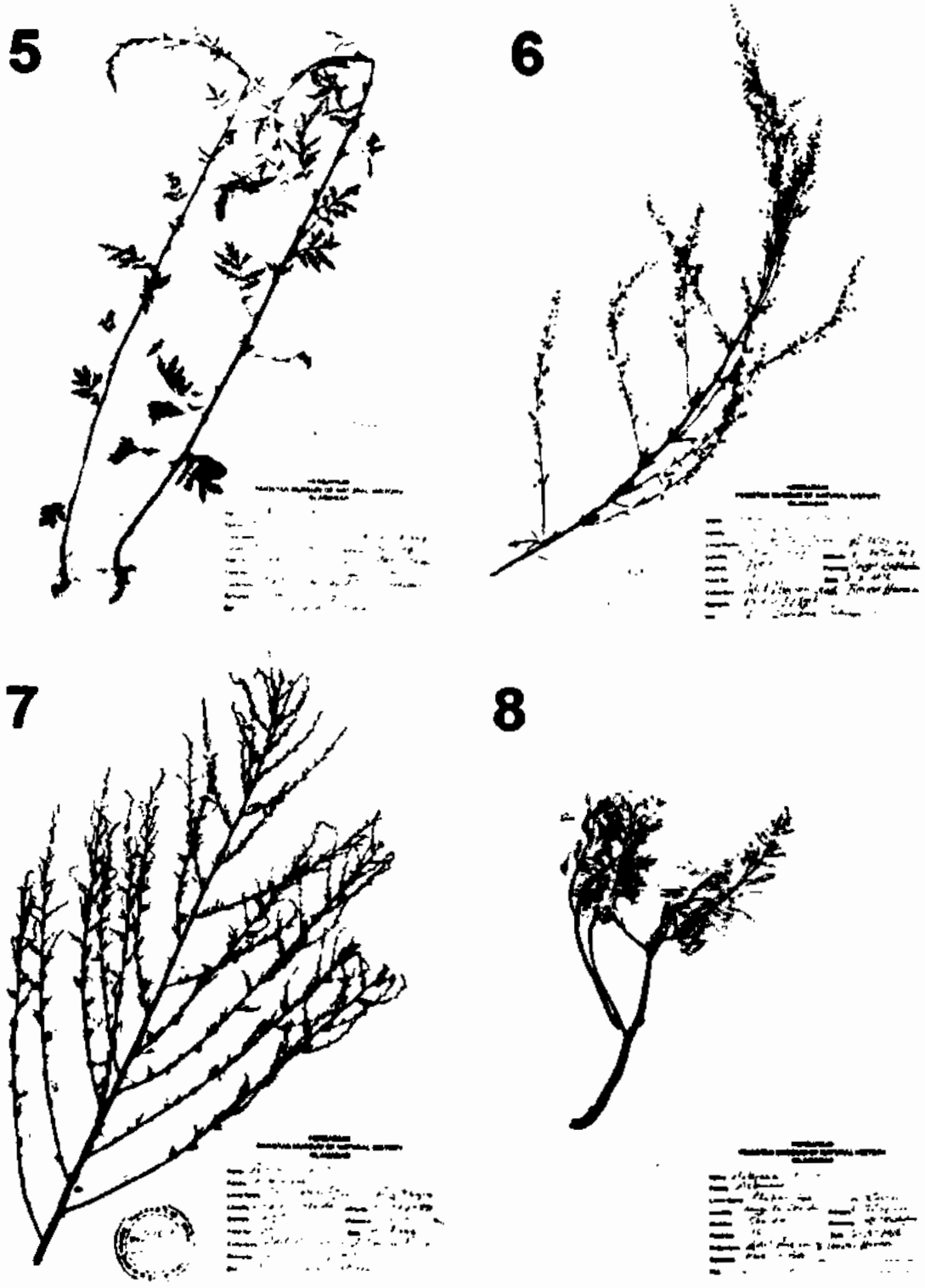

Plate 4.36. Continued... 5= *A. biennis*; 6= *A. campestris*; 7= *A. capillaris*; 8= *A. chinensis*

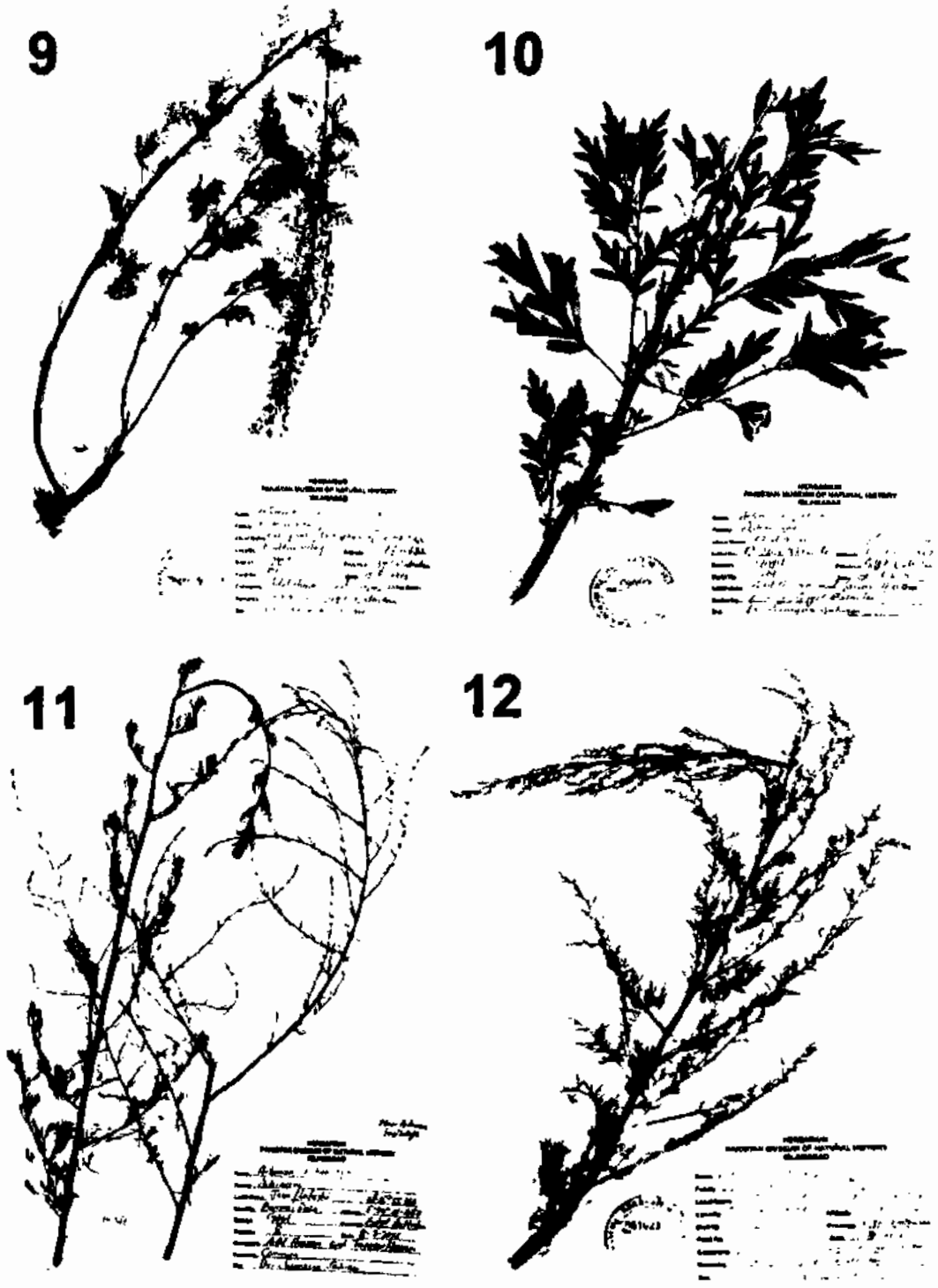

Plate 4.36. Continued... 9= *A. chamaemelifolia*; 10= *A. dubia*; 11= *A. sp.*-AD-H; 12= *A. gmelinii*

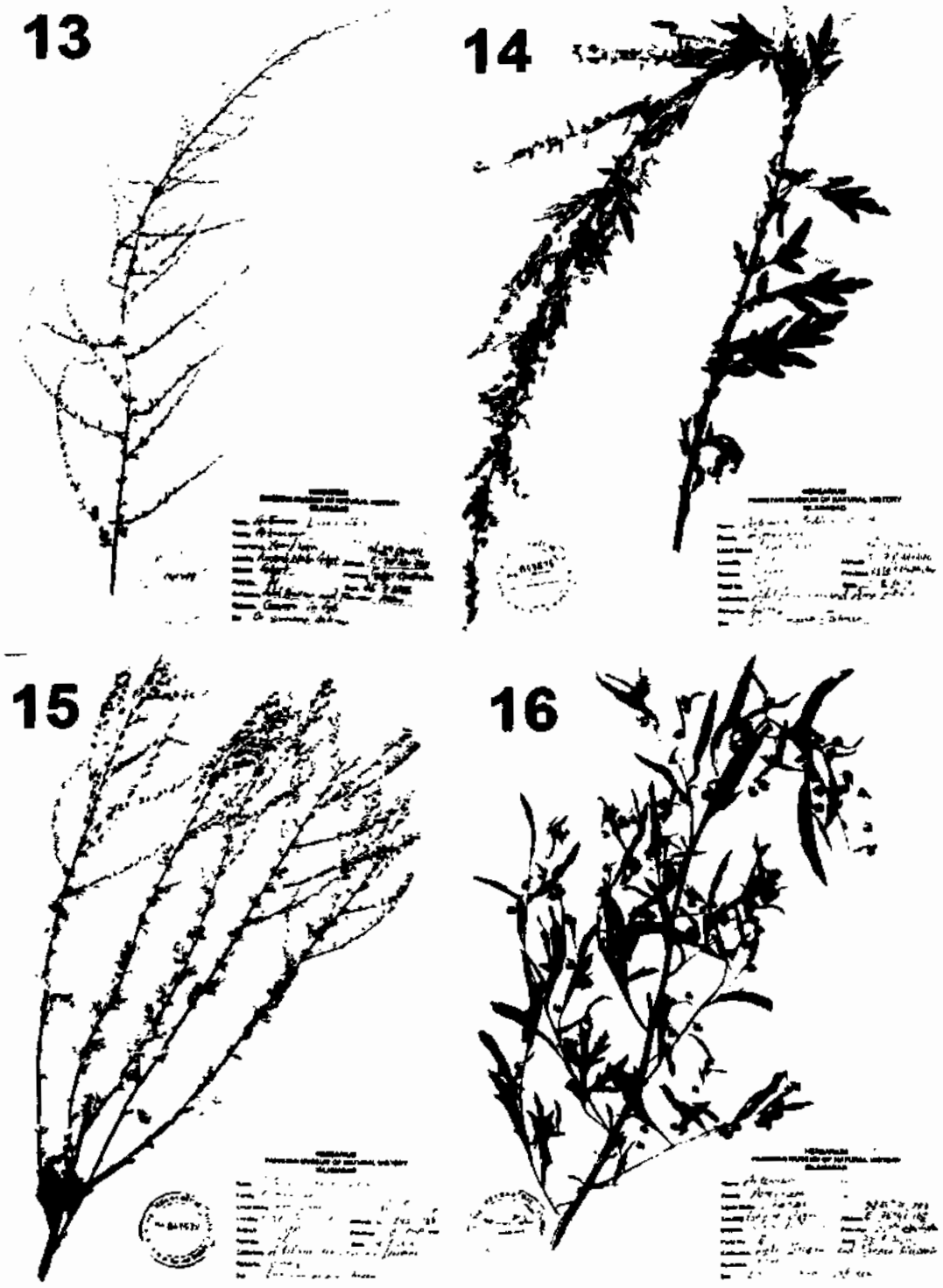

Plate 4.36. Continued... 13= *A. herba-alba*; 14= *A. indica*; 15= *A. maritima*; 16= *A. montana*

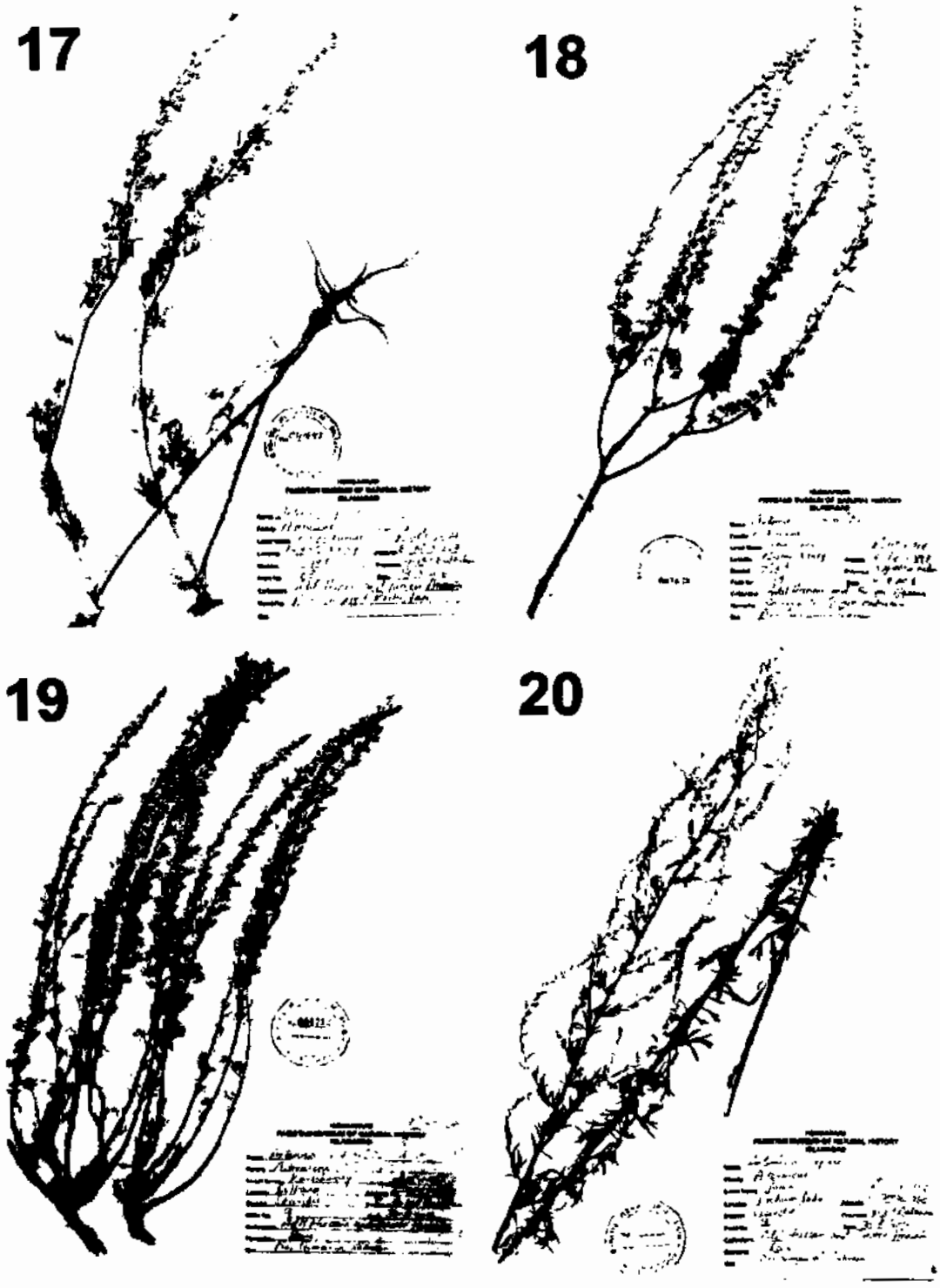

Plate 4.36. Continued... 17= *A. pontica*; 18= *A.rutifolia*; 19= *A. rutifolia sub sp.*; 20= *A. scoparia*

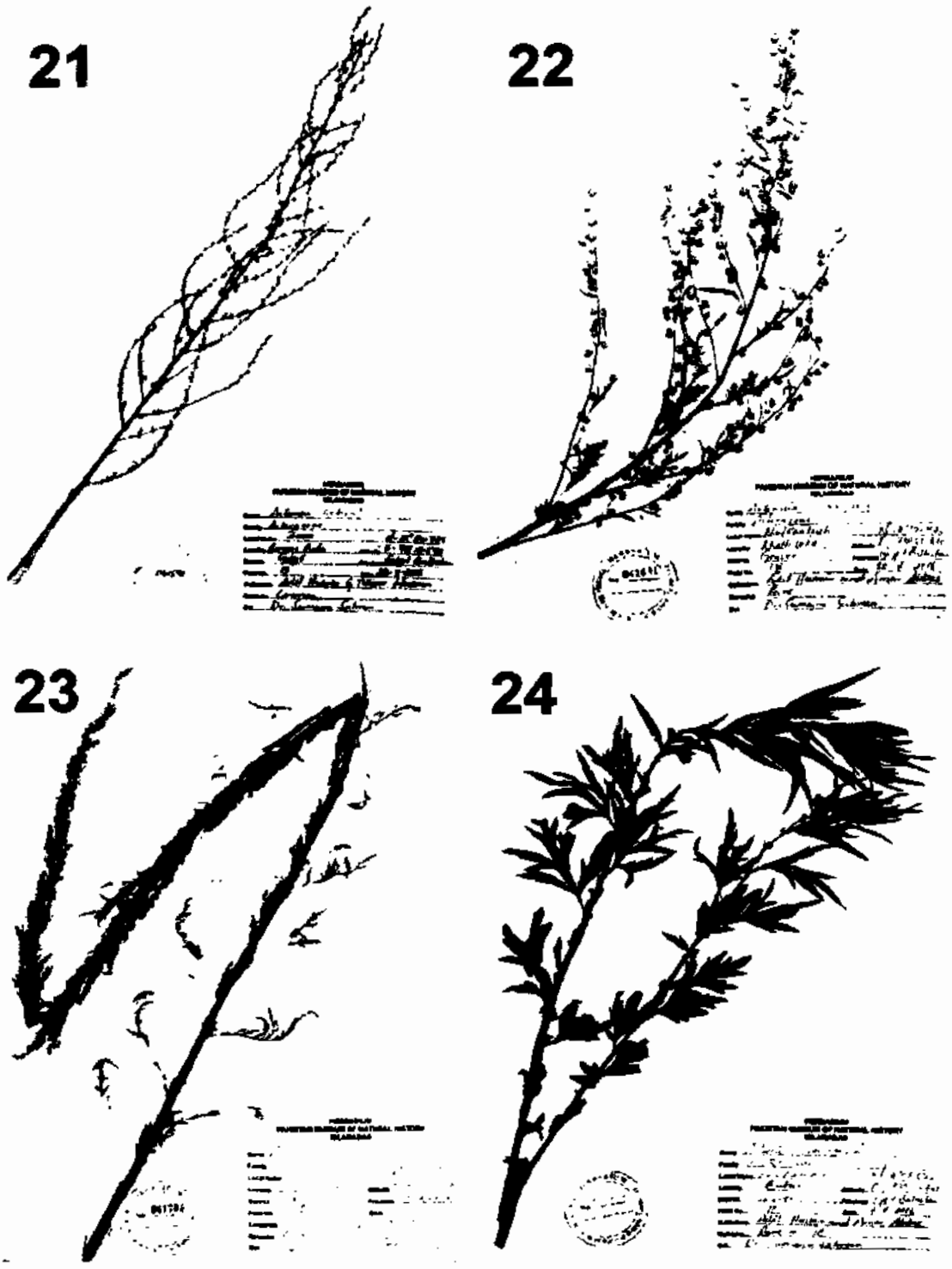

Plate 4.36. Continued... 21= *A. sieberi*; 22= *A. sieversiana*; 23= *A. tournefortiana*; 24= *A. verlotiorum*

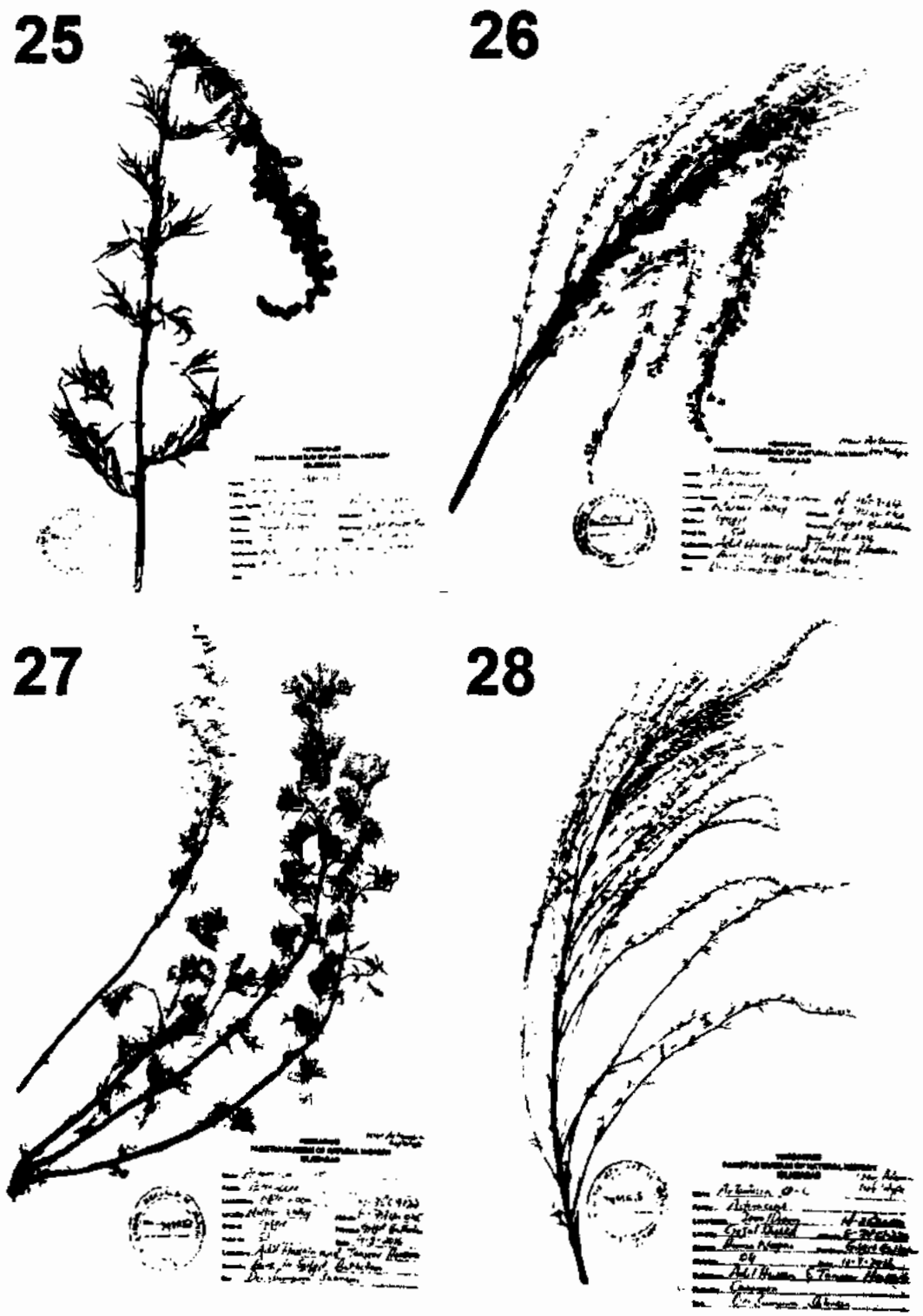

Plate 4.36. Continued... 25= *A. vulgaris*; 26= *A.* sp.–A; 27= *A.* sp.-B; 28= *A.* sp. -C

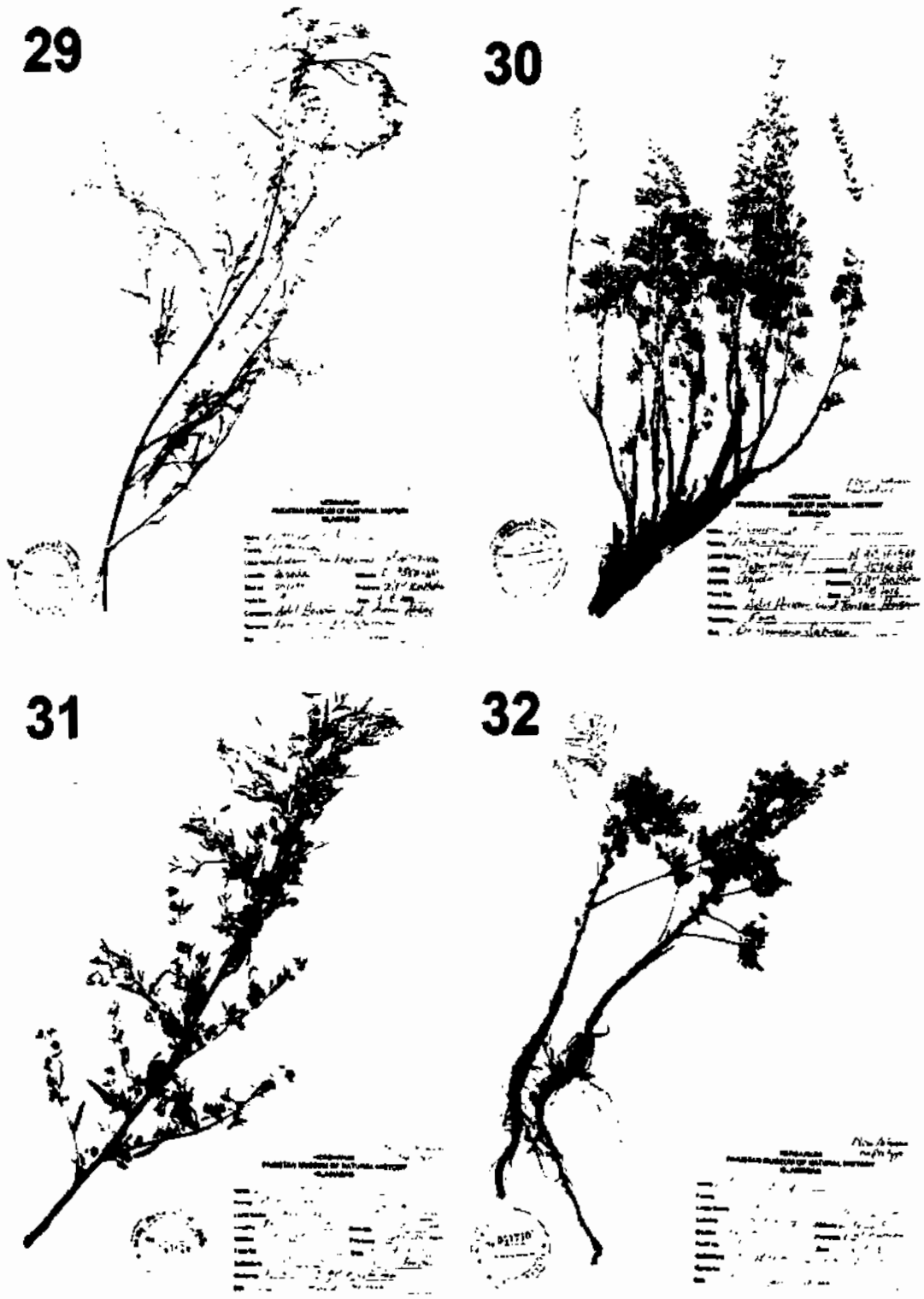

Plate 4.36. Continued... 29= *A.* sp.-D; 30= *A.* sp.-E; 31= *A.* sp.-F; 32= *A.* sp.-G

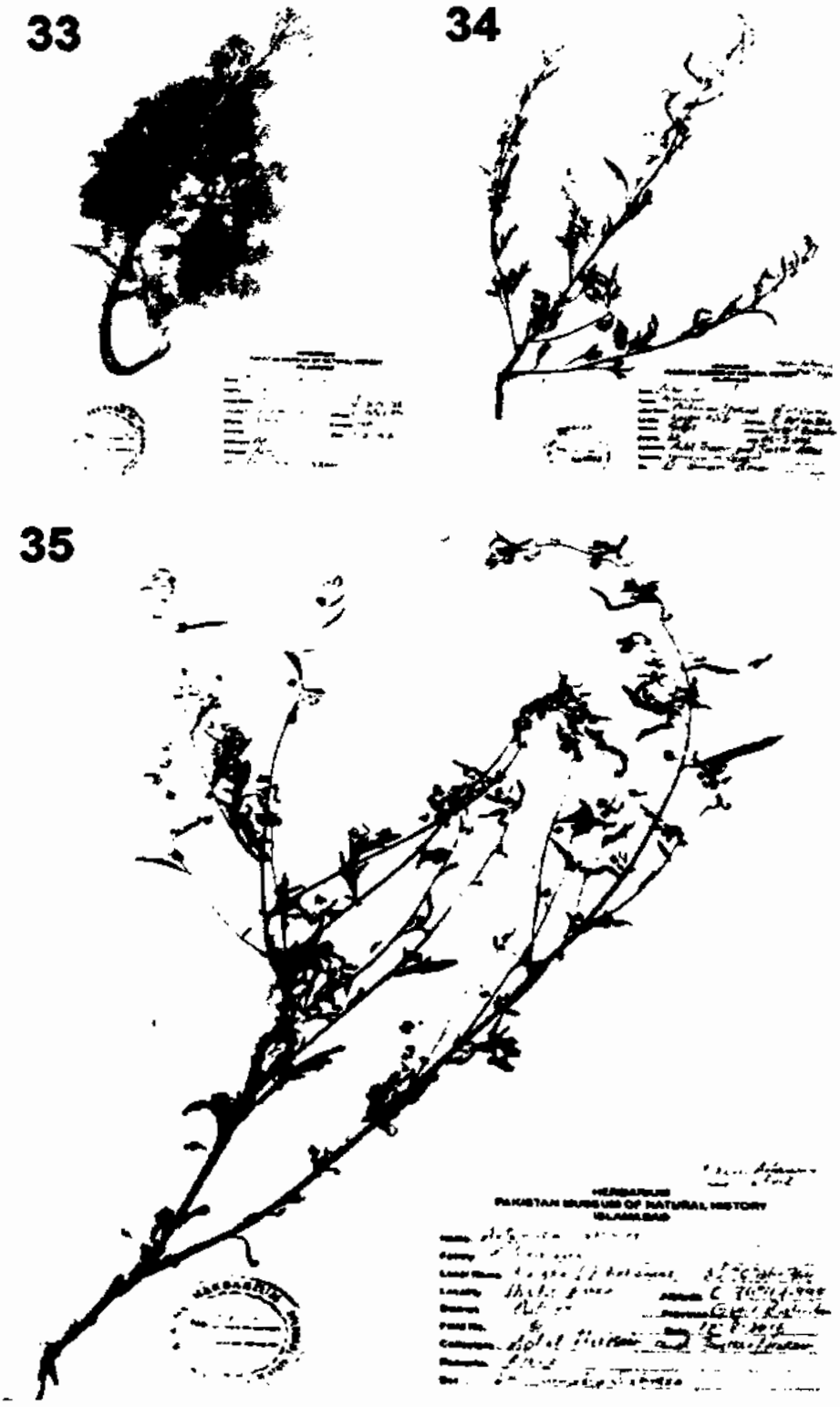

Plate 4.36. Continued... 33= *A. arborescens*; 34= *A.* sp.-I, 35= *A.* sp.-H

4.2. Morphological Phylogeny

Artemisia is a diverse genus on the basis of its morphological attributes. Species of *Artemisia* from Gilgit-Baltistan region of Pakistan also showed startling diversity. Few strong aromatic species include *A. annua, A. austriaca, A. arborescens, A. chamaemelifolia, A. gmelini, A. herba-alba, A. rutifolia,* and *A. maritima* while the rest of species were slightly aromatic.

Figure 4.4 represents the life cycles distribution (80% perennials, 10% biannuals and 10% annuals) and Figure 4.5 shows the life forms distribution of *Artemisia* species (35% Shrubby and 65% herbs) for the morphological analysis from Gilgit-Baltistan region of Pakistan.

Figure 4.6 shows the variations in plant height of different *Artemisia* species from Gilgit-Baltistan region of Pakistan. The maximum recorded plant height was from 100 to 250 cm in *A. tournefortiana* and *A. biennis* whilst the minimum plant height noticed was 10-30 cm in *A. herba-alba, A. chinensis, A. capillaris, A. rutifolia, A. austriaca* and *A. maritima.*

Figure 4.7 depicts the leaf petiole length variations of different *Artemisia* species. *A. annua, A. biennis, A. campestris, A. indica, A. scoparia, A. capillaris, A. tournrfourtiana, A. pontica* and *A. chamaemelifolia* were found with maximum petiole length from 3-10 cm while minimum leaf petiole length (0.5-1 cm) was recorded in *A. herba alba, A. maritima, A. arborescens, A. austriaca, A. sieberi, A. verlotiorum* and *A. vulgaris.* Few species leaves were shown to be sessile like *A. chinensis.*

Variations in the capitular length of different *Artemisia* species from Gilgit Baltistan region of Pakistan is given in Figure 4.8. Where, maximum length was noticed in *A. arborescens, A. chamaemelifolia,* and *A. pontica* (3-6 sq. mm) while the minimum length was found in *A. annua, A. capillaris, A. sieberi, A. scoparia* and *A. tournefortiana* (1-2 sq. mm).

Figures 4.9 interpret ray florets number of different *Artemisia* species. It has been said that the ray and disc florets have positive correlation with capitular diameter. In this study the maximum number of ray florets was found (5 to 20) in *A. biennis, A. arborescens, A. chinensis* and *A. pontica* whereas minimum number of ray florets was found (<5) in *A. campestris* and *A. rutifolia.* Other species including *A. maritima, A.*

sieberi and *A. herba-alba* lack the ray florets as shown in Figure 4.9. Maximum number of disc florets of different *Artemisia* species from Gilgit Baltistan region of Pakistan was shown in Figure 4.10. The maximum number of disc florets was found (30-45) in *A. biennis, A. capillaris, A. arborescens, A. chinensis, A. pontica, A. rutifolia, A. vulgaris and A. verlotiorum.* Minimum number of disc florets was found (5-10) in A. *maritima, A. herba-alba, A. austriaca, A. scoparia* and *A. sieberi.*

The variations of corolla length in ray and disc florets of different *Artemisia* species from Gilgit-Baltistan region of Pakistan are given in Figures 4.11 and 4.12 where the maximum corolla length of ray florets recorded was 2 mm in *A. annua, A. arborescens and A. austriaca, A. biennis, A. campestris, A. chamaemelifolia, A. chinensis, A. indica, A. pontica, A. tournefortiana, A. verlotiorum* and *A. vulgaris* while minimum corolla length of ray florets recorded was 0.5-1 mm in *A. gmelini, A. rutifolia, A. scoparia, A. montana* and *A. capillaris* as shown in Figure 4.11. Maximum corolla length of disc florets recorded was 1-2.5 mm in *A. austriaca,* and *A. montana.* While, the minimum corolla length of disc florets recorded was 0.6-1 mm in *A. indica,* and *A. rutifolia* as shown in Figure 4.12. The rest of species showed moderate corolla length of 1-2mm. Figure 4.13 gives information about variation in cypsela size of different *Artemisia* species from Gilgit-Baltistan region of Pakistan. Variation in color of cypsela was noticed from light to dark brown shades in the investigated *Artemisia* species.

Most of the cypselas possess a terminal scar and only few of the species were having a lateral scar. Maximum cypsela length recorded was 1-1.5 sq. mm in *A. annua, A. arborescens A. austriaca, A. chinensis, A, indica, A. maritima, A. montana* and *A. tournefortiana.* While minimum cypsela length recorded was 0.5-0.9 sq. mm in rest of the *Artemisia* species as shown in Figure 4.13. Few species have special morphology and can be differentiated easily form other *Artemisia* species. For example, *A. austriaca, A. rutifolia,* have matchless plant morphology with dense visible silvery hairs in its leaves and stem. *A. chinensis* have silvery leaves with three round teeth. Few species have simple leaves while the rest of *Artemisia* species have dissected leaves. For a cladistic analysis of *Artemisia* species collected from Gilgit-Baltistan region of Pakistan, A strict consensus cladogram (Figure. 4.14) was constructed based on

the data matrix (Table 4.2) obtained from the morphological traits of different *Artemisia* species given in Table 3.2.

Figure 4.14 also represents the comparison of this morphological work with classical classification of the genus *Artemisia* by Ling (1991), Torell *et al.*, (1999), D'Andrea *et al.*, (2003), Pellicer *et al.*, (2010) and Garcia *et al.*, (2011). Based on the results of cladogram, the genus *Artemisia* is divided into four major clades. Subgenus *Artemisia* and *Absinthium* were dispersed between the clades of other subgenera and appeared as a polyphyletic. All the remaining subgenera were found to be monophyletic. Subgenus *Seriphidium* grouped with the *Artemisia* clade which authenticates its recombination within the genus *Artemisia*.

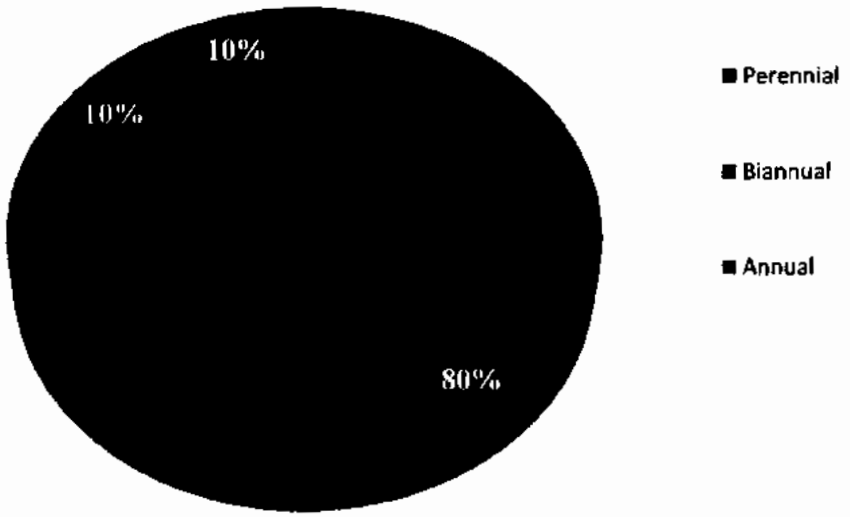

Figure 4.4. Life cycles distribution of *Artemisia* for morphological study from Gilgit-Baltistan, Pakistan.

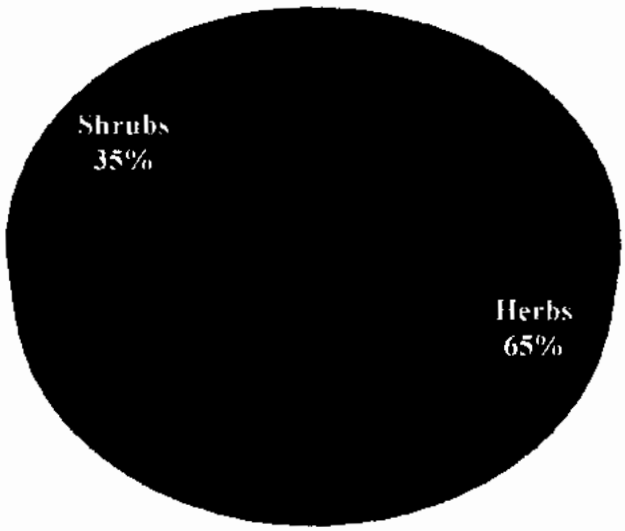

Figure 4.5. Life form distribution of *Artemisia* for morphological study from Gilgit-Baltistan, Pakistan.

Table 4.2. Data matrix for cladistic analysis in *Artemisia* species on the basis of different morphological attributes (Character and Character states are described in table 3.2)

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
A. annua	2	0	1	0	0	1	0	1	1	0	0	1	2	2	0	0	0	0	0	0	0	0
A arborescens	0	1	1	0	0	0	0	0	0	0	1	0	3	2	0	0	1	1	0	1	0	0
A. austriaca	0	1	1	0	0	1	0	1	1	0	1	0	2	1	1	0	1	1	1	1	0	0
A biennis	1	0	0	1	0	1	0	0	0	1	1	1	1	3	4	0	0	0	0	0	0	1
A. campestris	0	0	0	1	0	1	0	0	0	1	1	1	1	1	3	1	1	1	1	0	1	1
A. chamaemelifolia	0	0	1	0	0	1	0	0	0	0	1	1	2	1	4	0	0	1	0	0	0	0
A. chinensis	0	1	1	1	0	0	0	0	0	1	1	1	0	1	4	0	1	1	1	3	0	0
A. gmelinii	0	0	1	0	0	1	0	0	0	1	1	1	1	2	4	0	1	1	0	2	0	0
A.capillaris	0	0	0	1	0	0	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	0
A. herba alba	0	1	0	0	1	1	1	0	1	0	1	0	1	0	1	1	1	1	0	0	0	0
A. indica	0	0	0	1	0	1	0	0	0	1	1	1	1	2	0	0	1	0	0	1	0	1
A. maritima	0	1	0	0	1	1	1	0	1	0	1	1	1	0	0	1	1	1	0	0	0	0
A. montana	0	0	1	0	0	1	1	0	0	0	1	0	1	3	0	0	0	0	0	1	0	0
A. pontica	0	0	1	0	0	1	1	0	0	0	1	1	2	2	4	1	1	1	0	1	0	0
A. rutifolia	0	1	1	0	1	1	0	1	1	0	1	1	2	1	1	0	1	1	0	2	0	0
A. scoparia	1	0	1	1	0	1	0	1	1	1	1	0	0	1	6	1	1	1	0	1	0	0
A. sieberi	0	1	0	0	1	1	0	1	1	0	1	1	2	0	4	0	1	1	0	2	1	1
A tournefortiana	2	0	1	1	0	1	0	1	1	0	0	1	1	3	6	0	0	0	0	0	0	1
A. verlotiorum	0	0	0	1	0	1	0	0	1	0	0	1	2	2	4	0	1	0	0	2	0	0
A. vulgaris	0	0	0	1	0	1	0	0	0	0	1	1	2	3	5	0	1	0	0	1	0	1
OUT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4.2. Continued

Taxa	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
A. annua	0	0	0	2	1	1	1	1	1	1	0	2	3	0	3	2	2	0	1	1	1	0
A. arborescens	1	0	0	2	0	0	0	0	0	1	2	0	1	0	2	1	2	0	0	1	1	0
A. austriaca	0	0	1	2	0	0	0	1	1	0	2	0	3	0	2	0	2	0	0	1	0	0
A. biennis	2	0	0	2	1	1	0	0	0	0	0	1	2	1	3	2	2	0	1	1	1	0
A. campestris	2	0	0	1	1	1	1	1	1	0	1	1	1	0	1	2	1	1	1	1	0	0
A. chamaemelifolia	2	0	0	2	1	1	1	1	1	1	2	0	3	0	3	2	2	0	1	1	1	1
A. chinensis	0	0	0	2	1	1	0	1	1	0	3	0	2	0	1	0	0	1	0	1	0	0
A. capillaris	0	0	0	1	0	1	0	1	1	0	2	2	3	0	1	2	2	0	1	1	0	1
A. gmelini	0	0	1	1	1	1	1	1	1	1	2	1	2	0	0	0	1	1	1	1	1	0
A. herba alba	0	0	0	2	1	0	1	1	1	1	3	1	3	1	0	0	1	0	1	1	1	0
A. indica	1	0	0	2	0	1	1	1	1	0	2	1	1	1	2	1	1	1	0	1	0	0
A. maritima	0	0	1	2	1	0	1	1	1	1	3	1	2	1	0	1	1	0	0	1	1	0
A. montana	1	1	0	2	0	0	0	0	0	1	3	1	2	0	3	0	2	0	1	0	1	0
A. pontica	1	0	0	2	0	1	0	0	1	0	2	0	1	1	2	0	1	0	0	1	0	0
A. rutifolia	0	0	1	1	1	1	0	1	1	1	2	1	1	1	2	1	2	0	1	1	1	0
A. scoparia	2	0	0	1	1	1	1	1	1	0	2	2	3	0	2	2	2	1	1	1	0	0
A. sieberi	0	0	1	1	1	1	0	1	0	1	3	1	3	1	0	1	1	1	0	1	1	0
A. tournefortiana	0	0	0	1	1	0	1	1	0	1	1	2	3	0	2	2	2	1	1	1	1	0
A. verlotorum	1	1	1	2	0	1	0	0	0	0	3	1	3	1	3	0	2	1	1	1	1	0
A. vulgaris	0	1	1	2	0	1	1	0	0	0	3	1	3	1	1	0	2	1	0	1	1	0
OUT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4.2. Continued

Taxa	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66
A. annua	3	1	2	1	2	1	1	2	0	3	1	0	0	0	0	0	0	1	1	1	0	0
A arborescens	2	0	0	1	0	1	3	1	0	1	1	3	0	0	1	0	0	1	1	1	0	0
A. austriaca	0	0	1	1	2	1	0	0	0	4	0	3	0	0	0	0	0	0	1	1	0	0
A. biennis	3	1	1	0	0	1	0	1	0	1	1	0	0	0	0	2	0	1	0	0	0	0
A campestris	2	1	1	0	3	1	0	0	0	3	1	0	0	1	0	0	0	1	0	0	0	0
A. chamaemelifolia	3	1	2	0	1	1	0	2	0	1	1	0	0	0	0	0	1	0	0	0	0	0
A. chinensis	3	1	2	2	0	1	3	2	0	0	1	0	0	1	1	0	1	0	1	1	0	0
A. gmelini	3	1	1	0	1	2	0	0	0	3	1	2	0	0	1	0	0	1	0	0	0	0
A.capillaris	2	1	2	0	1	2	0	0	0	3	1	3	0	1	1	1	0	0	0	0	0	0
A herba alba	2	1	1	1	4	3	4	4	1	4	1	0	0	0	1	1	1	0	1	1	0	0
A. indica	0	1	0	0	2	1	0	0	0	3	2	2	1	0	1	2	0	0	1	1	0	0
A. maritima	2	1	1	1	4	3	4	4	1	4	1	1	0	0	1	1	1	0	1	1	0	0
A. montana	2	1	1	0	2	2	0	0	0	3	0	3	0	0	0	1	0	1	1	0	0	0
A. pontica	0	1	0	0	1	1	1	0	0	1	2	0	0	0	0	0	1	0	0	0	0	0
A. rutifolia	0	1	2	1	3	2	0	0	0	1	2	0	1	0	0	0	0	1	0	1	0	0
A. scoparia	3	1	2	0	2	2	0	2	0	4	1	0	0	0	1	0	0	0	0	0	0	0
A. sieberi	3	1	1	2	4	3	4	4	1	4	1	0	0	0	1	1	0	1	0	1	0	0
A. tournefortiana	2	1	1	0	1	1	0	0	0	3	1	3	0	0	1	0	0	1	1	1	0	0
A. verlotorum	0	1	2	0	2	1	0	1	0	1	1	0	0	0	1	0	0	1	0	0	0	0
A. vulgaris	2	1	1	0	2	1	0	0	0	0	1	3	1	0	1	1	0	0	0	0	0	0
OUT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

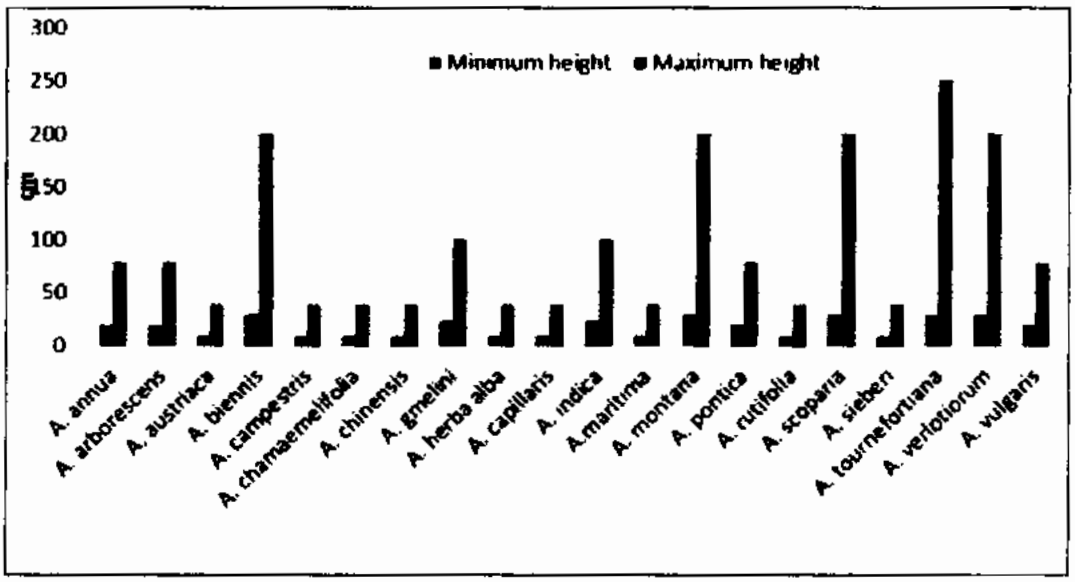

Figure 4.6. Variation in plant height *Artemisia* species from Gilgit-Baltistan, Pakistan.

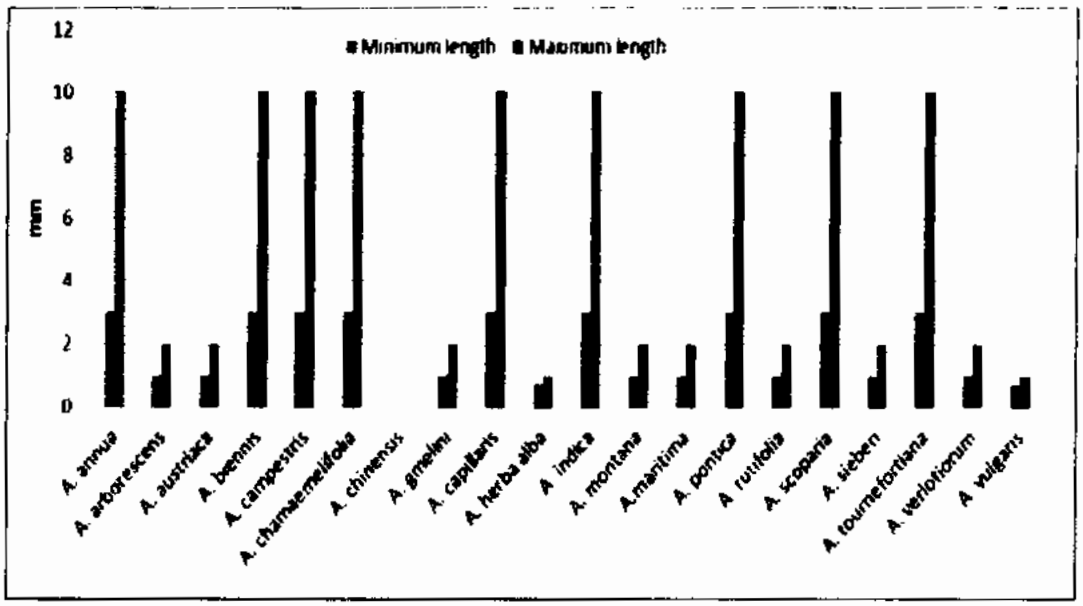

Figure 4.7. Variation in leaf petiole length of *Artemisia* species from Gilgit-Baltistan, Pakistan.

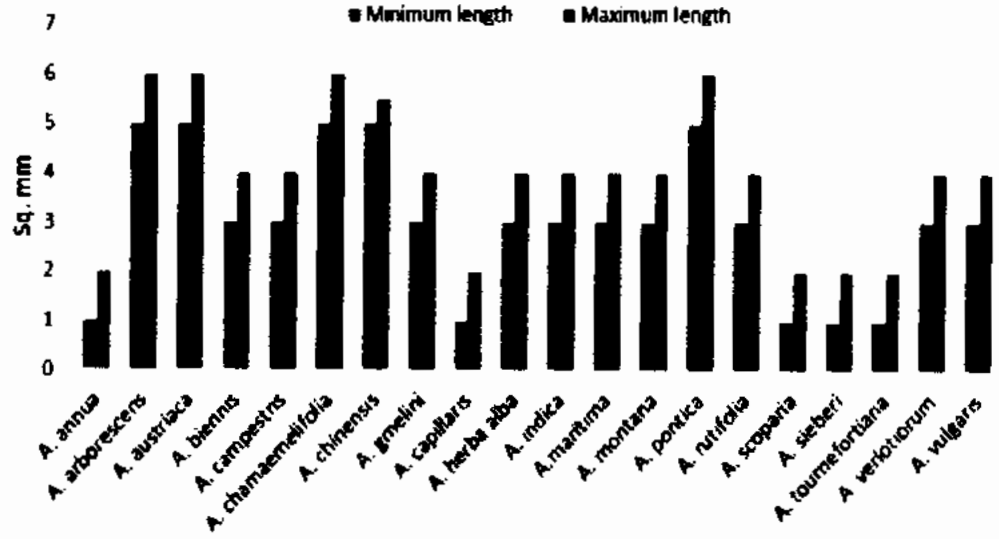

Figure 4.8. Variation in capitulum length of *Artemisia* species from Gilgit-Baltistan, Pakistan.

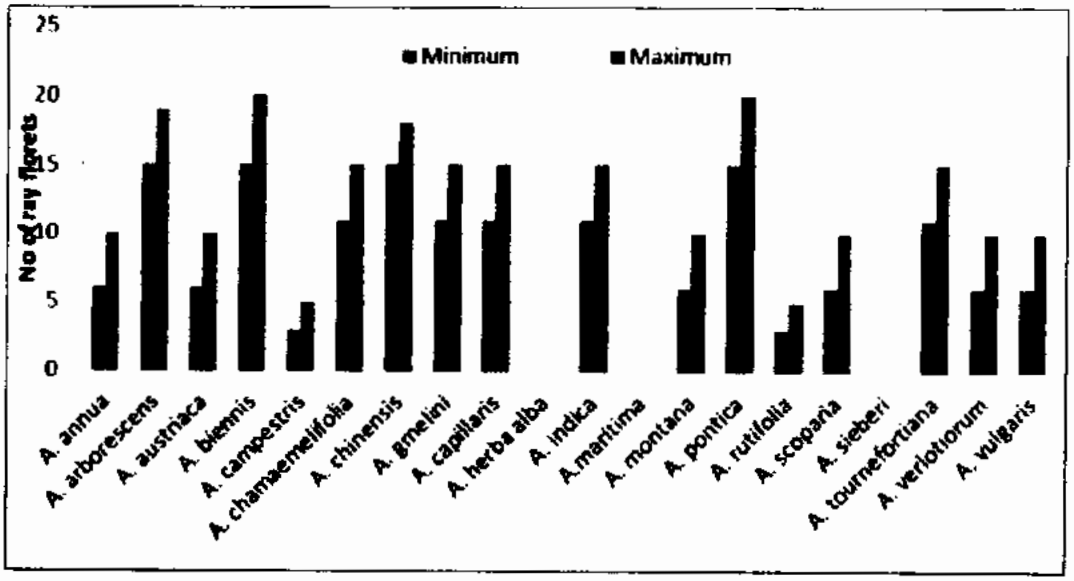

Figure 4.9. Variation in number of ray florets of *Artemisia* species from Gilgit-Baltistan, Pakistan.

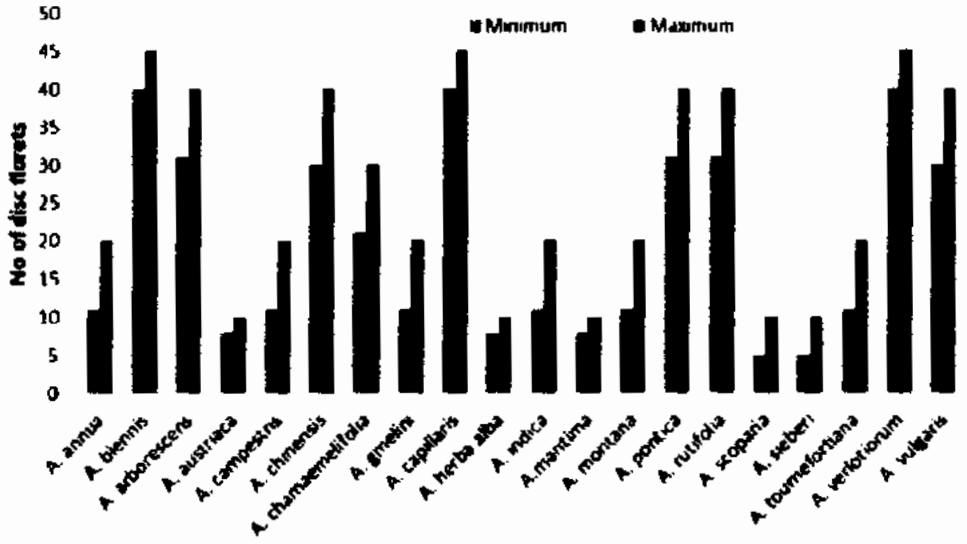

Figure 4.10. Variation in number of disc florets of *Artemisia* species from Gilgit-Baltistan, Pakistan.

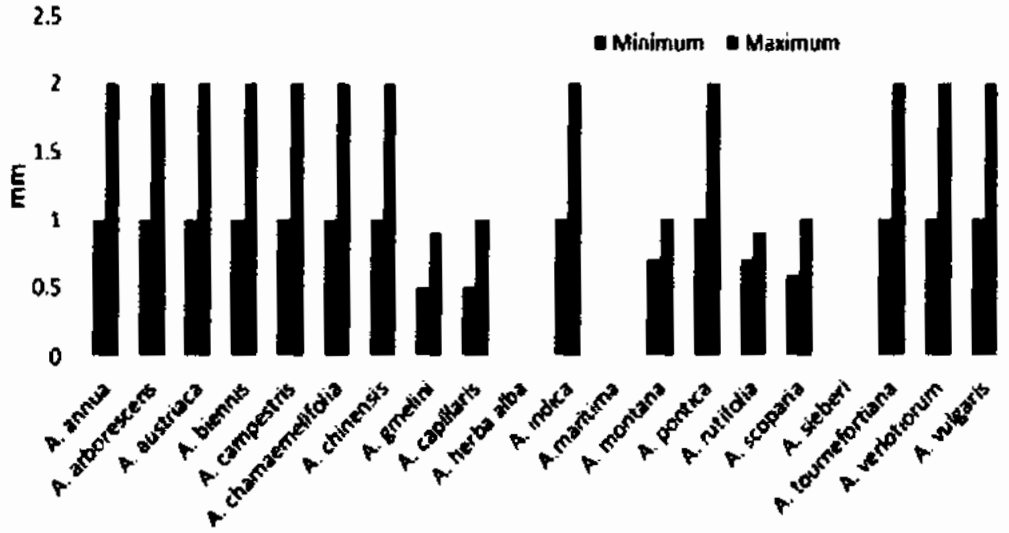

Figure 4.11. Variation in corolla length in ray florets of *Artemisia* species from Gilgit-Baltistan, Pakistan.

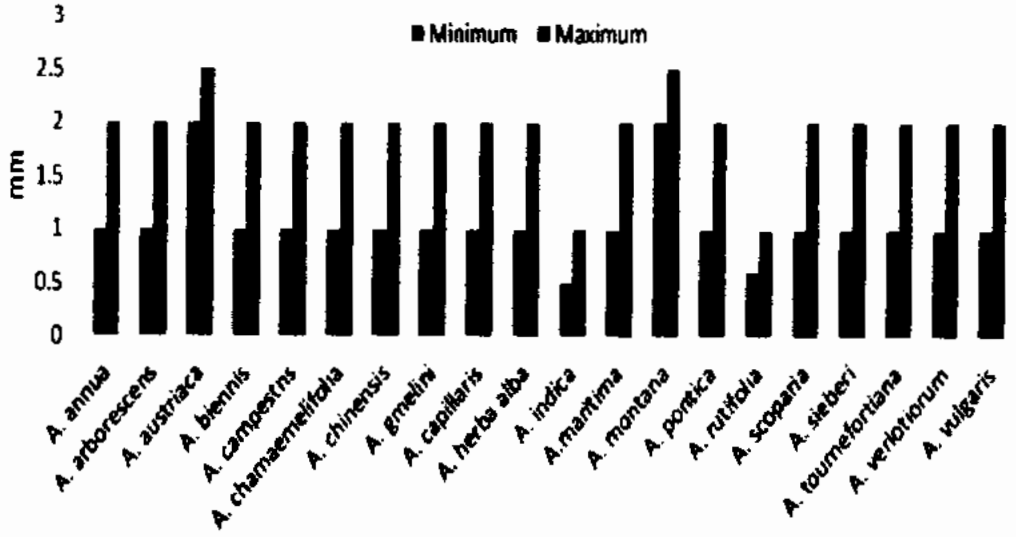

Figure 4.12. Variation in corolla length in disc florets of *Artemisia* species from Gilgit-Baltistan, Pakistan.

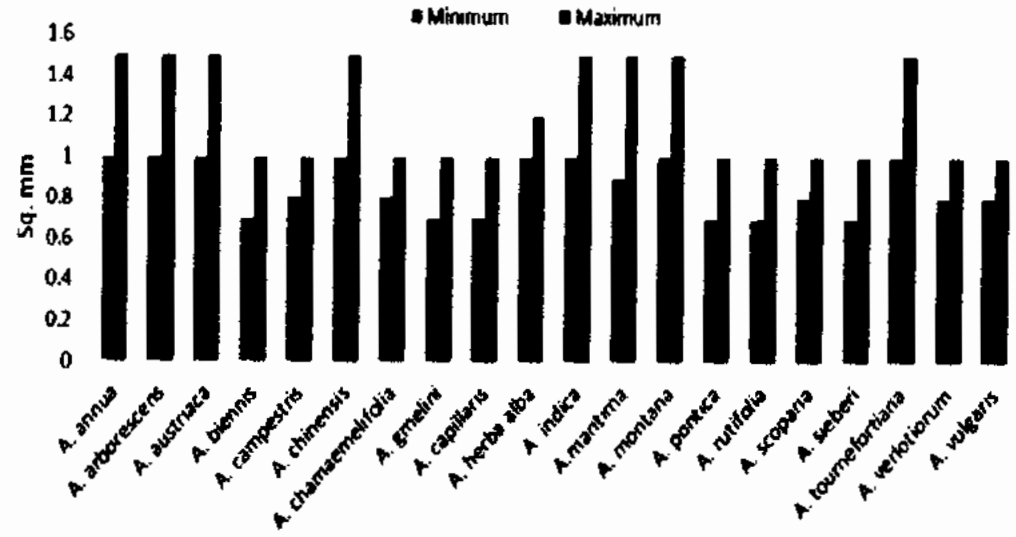

Figure 4.13. Variation in cypsela size (length x width) of *Artemisia* species from Gilgit-Baltistan, Pakistan.

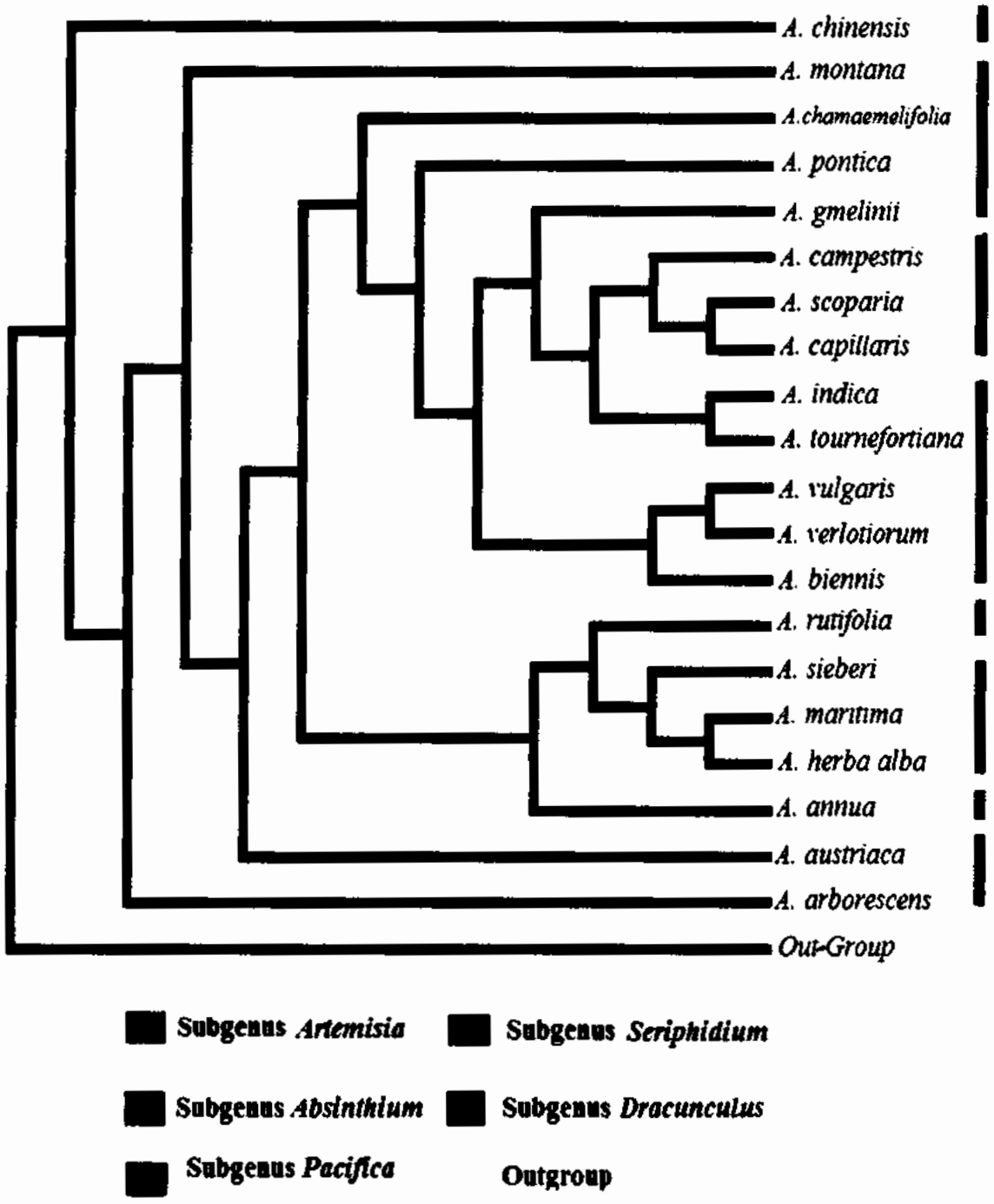

Figure 4.14. Strict consensus cladogram based on floral morphology of genus *Artemisia* from Gilgit-Baltistan Pakistan based on morphological traits given in Table 3.2 using maximum parsimony method. Labels symbolises the traditional infrageneric classification of *Artemisia* genus on molecular basis given by Ling (1991), Korenkeven *et al.*, (1999), Torell *et al.*, (1999), D'Andrea *et al.*, (2003), Pellicer *et al.*, (2010) and Garcia *et al.*, (2011).

4.3. Leaf epidermal anatomy

4.3.1. Epidermis and stomata

The studied *Artemisia* species showed variations in their leaf epidermal anatomical attributes, such as the epidermal cells structures and types of stomata.

Epidermal cells investigated among studied *Artemisia* species were varied from polygonal to elongate and irregular in shape, while smooth and wavy in margins as shown in Plate 4.37 and 4.38. Some *Artemisia* species like *A.* sp.AD-H, *A. maritima* and *A. herba alba* showed elongated smooth walls of cells as shown in figure 4.37-C, D, O. Few *Artemisia* species like *A. argyi*, *A. pontica* and *A. montana* showed polygonal shape with smooth walled cells (Plate 4.37-B, P, R). Other species showed irregular shape with wavy walls. (Plate 4.37-A, E, F, G, H, I, J, K, L, M, N, Q) (Plate 4.38-A, B, C, D, E, F, G).

The qualitative and quantitative attributes of all the epidermal cells of investigated *Artemisia* species based on SEM and LM investigations, were given in Table 4.3 and shown in Plate 4.37 and 4.38.

All the species had stomata on both surfaces, particularly on the abaxial epidermis. In this study, total five types of stomata were found in 17 different species of *Artemisia*. These types of stomata comprised of anomocytic, anisocytic, anomotetracytic, diacytic and paracytic. The details based on SEM observations of these stomata types are provided in Table 4.4 and shown in Plate 4.39.

A. chinensis (Plate 4.39-L) and *A. gmelinii* (Plate 4.39-N) showed Anomotetracytic type of stomata. Similarly, Diacytic types of stomata were found in *A. chamaemelifolia* (Plate 4.39-G). Paracytic types of stomata are only noticed in *A.* sp. AD-H, and *A. campestris* respectively (Plate 4.39-C, F).

Anisocytic types of stomata were also noticed in *A. pontica, A. vulgaris* and *A. verlotiorum* (Plate 4.39-O, P, I). Anomocytic type of stomata was common in all the rest of the species of *Artemisia* as shown in Plate 4.39.

Table 4.3. Foliar epidermal cells characteristics of different *Artemisia* species collected from different regions of Gilgit-Baltistan, Pakistan.

Artemisia spp.	Surface	Shape	Margin	Length µm	Width µm
A. annua	AB	Irregular	Wavy	36.11	14.21
	AD	Irregular	Wavy	41.87	15.00
A. austriaca	AB	Irregular	Wavy	40.30	11.62
	AD	Irregular	Wavy	41.27	13.28
A. chinensis	AB	Irregular	Wavy	30.23	9.84
	AD	Irregular	Wavy	36.94	14.24
A. campestris	AB	Irregular	Wavy	49.12	12.82
	AD	Irregular	Wavy	34.78	13.05
A. dubia	AB	Irregular	Wavy	42.19	13.55
	AD	Irregular	Wavy	56.11	22.64
A. sp. AD-H	AB	Elongated	Smooth	39.31	11.58
	AD	Elongated	Smooth	59.51	14.90
A. chamemelifolia	AB	Irregular	Wavy	75.38	21.79
	AD	Irregular	Wavy	79.00	22.60
A. argyi	AB	Polygonal	Smooth	32.78	33.69
	AD	Polygonal	Smooth	23.73	15.59
A. gmelinii	AB	Irregular	Wavy	20.44	12.53
	AD	Irregular	Wavy	28.04	10.89
A. herba-alba	AB	Elongated	Smooth	64.02	7.58
	AD	Elongated	Smooth	71.27	8.87
A. indica	AB	Irregular	Wavy	47.40	14.51
	AD	Irregular	Wavy	58.13	23.60
A. montana	AB	Polygonal	Smooth	30.69	17.10
	AD	Polygonal	Smooth	34.41	34.74
A. maritima	AB	Elongated	Smooth	61.68	7.38
	AD	Elongated	Smooth	41.74	3.96
A. pontica	AB	Polygonal	Smooth	26.47	20.30
	AD	Polygonal	Smooth	23.12	14.16
A. rutifolia	AB	Irregular	Wavy	36.94	14.37
	AD	Irregular	Wavy	49.34	13.09
A. scoparia	AB	Irregular	Wavy	32.61	19.57
	AD	Irregular	Wavy	30.69	14.75
A. tournefortiana	AB	Irregular	Wavy	29.70	12.17
	AD	Irregular	Wavy	39.43	16.64
A. verlotiorum	AB	Irregular	Wavy	46.37	31.97
	AD	Irregular	Wavy	58.22	29.30
A. vulgaris	AB	Irregular	Wavy	36.26	15.44
	AD	Irregular	Wavy	44.02	15.50

AB= Abaxial, AD= Adaxial

Table 4.4. Characteristics of stomata in different *Artemisia* species collected from different regions of Gilgit-Baltistan, Pakistan.

Artemisia spp.	Shape	Stomata Type			Guard cell	
			Length µm	Width µm	Length µm	Width µm
A. annua	AB	Anomocytic	20.24	16.61	17.52	6.47
	AD	Anomocytic	23.39	17.18	20.79	6.76
A. austriaca	AB	Anomocytic	25.59	29.19	22.36	12.11
	AD	Anomocytic	17.32	10.36	14.57	4.13
A. chinensis	AB	Anomotetracytic	26.98	19.76	18.28	8.5
	AD	Anomotetracytic	25.48	18.69	17.05	7.07
A. campestris	AB	Paracytic	24.41	17.34	22.09	7.21
	AD	Paracytic	30.18	21.67	25.30	9.07
A. sp. AD-H	AB	Paracytic	23.17	24.88	22.40	10.38
	AD	Paracytic	33.85	24.02	24.30	10.37
A. chamemelifolia	AB	Diacytic	32.28	21.52	26.60	8.95
	AD	Diacytic	40.71	23.17	35.47	9.34
A. argyi	AB	Anomocytic	34.29	20.86	28.65	9.75
	AD	Anomocytic	32.44	20.65	25.42	10.60
A. gmelinii	AB	Anomotetracytic	39.92	25.48	24.76	9.14
	AD	Anomotetracytic	17.32	10.36	14.57	4.13
A. indica	AB	Anomocytic	20.84	19.06	26.10	9.12
	AD	Anomocytic	30.17	22.52	27.86	19.44
A. montana	AB	Anomocytic	25.41	23.73	8.37	10.34
	AD	Anomocytic	25.41	23.73	23.84	8.37
A. maritima	AB	Anisocytic	29.35	18.39	21.65	9.76
	AD	Anisocytic	22.35	15.54	20.56	8.37
A. pontica	AB	Anisocytic	25.89	19.71	22.77	6.48
	AD	Anisocytic	23.75	20.84	21.32	8.31
A. herba-alba	AB	Anomocytic	15.60	10.24	13.67	4.95
	AD	Anomocytic	30.20	21.46	27.04	10.20
A. scoparia	AB	Anomocytic	25.87	22.02	22.30	7.93
	AD	Anomocytic	28.21	20.44	18.63	8.26
A. tournefortiana	AB	Anomocytic	30.80	19.23	22.65	6.53
	AD	Anomotetracytic	29.92	20.78	26.46	7.12
A. verlotiorum	AB	Anisocytic	25.70	15.90	23.30	10.11
	AD	Anisocytic	24.90	15.75	21.31	8.28
A. vulgaris	AB	Anisocytic	19.20	14.30	14.70	7.7
	AD	Anisocytic	18.24	14.48	15.03	6.24

AB= Abaxial, AD= Adaxial

Table 4.4. Continued...

Artemisia spp.		Stomata aperture		No. of subsidiary cells	Stomata complex	
		Length µm	Width µm		Length µm	Width µm
A. annua	AB	9.52	4.42	5	43.69	42.39
	AD	12.18	2.79	5	53.01	33 25
A. austriaca	AB	19.63	4.70	---	Not	differentiated
	AD	11.92	2.77	---	Not	differentiated
A. chinensis	AB	10.49	1.87	4-5	65.06	43.27
	AD	14.39	2.49	4	46.97	50.29
A. campestris	AB	15.01	2.86	2	39.39	28.10
	AD	16.77	3.80	2	65.60	50.26
A. sp. AD-H	AB	12.50	4.30	2	44.51	35.85
	AD	11.19	4.33	2	46.03	41.73
A. chamemelifolia	AB	18.77	4.13	2	65.80	51.00
	AD	21.80	4.87	2	52.60	40.92
A. argyi	AB	23.60	3.03	---	Not	differentiated
	AD	24.40	6.01	---	Not	differentiated
A. gmelinii	AB	17.04	4.57	---	Not	differentiated
	AD	11.92	2.77	5-6	48.51	42.71
A. indica	AB	23.98	4.24	4	60.12	45.52
	AD	15.5	5.56	5-6	50.42	41.81
A. montana	AB	4.89	3.30	5	35.10	36.40
	AD	10.34	4.89	5	36.01	37.38
A. maritima	AB	13.40	4.86	3	34.54	29.59
	AD	12.65	6.87	3	34.54	29.59
A. pontica	AB	14.34	4.55	3	40.15	39.20
	AD	14.46	3.22	3	42.70	38.20
A. herba-alba	AB	9.37	1.28	4	29.36	29.85
	AD	17.72	2.19	4-5	59.16	49.59
A. scoparia	AB	16.73	3.38	4	73.01	54.73
	AD	18.90	3.34	4	73.54	64.04
A. tournefortiana	AB	16.35	5.62	4	61.76	56.98
	AD	15.33	3.65	4	59.50	49.59
A. verlotiorum	AB	17.30	4.10	---	Not	differentiated
	AD	16.57	2.58	---	Not	differentiated
A. vulgaris	AB	4.12	2.31	3	54.60	50.15
	AD	10.69	1.51	3	55.30	52.40

AB= Abaxial, AD= Adaxial

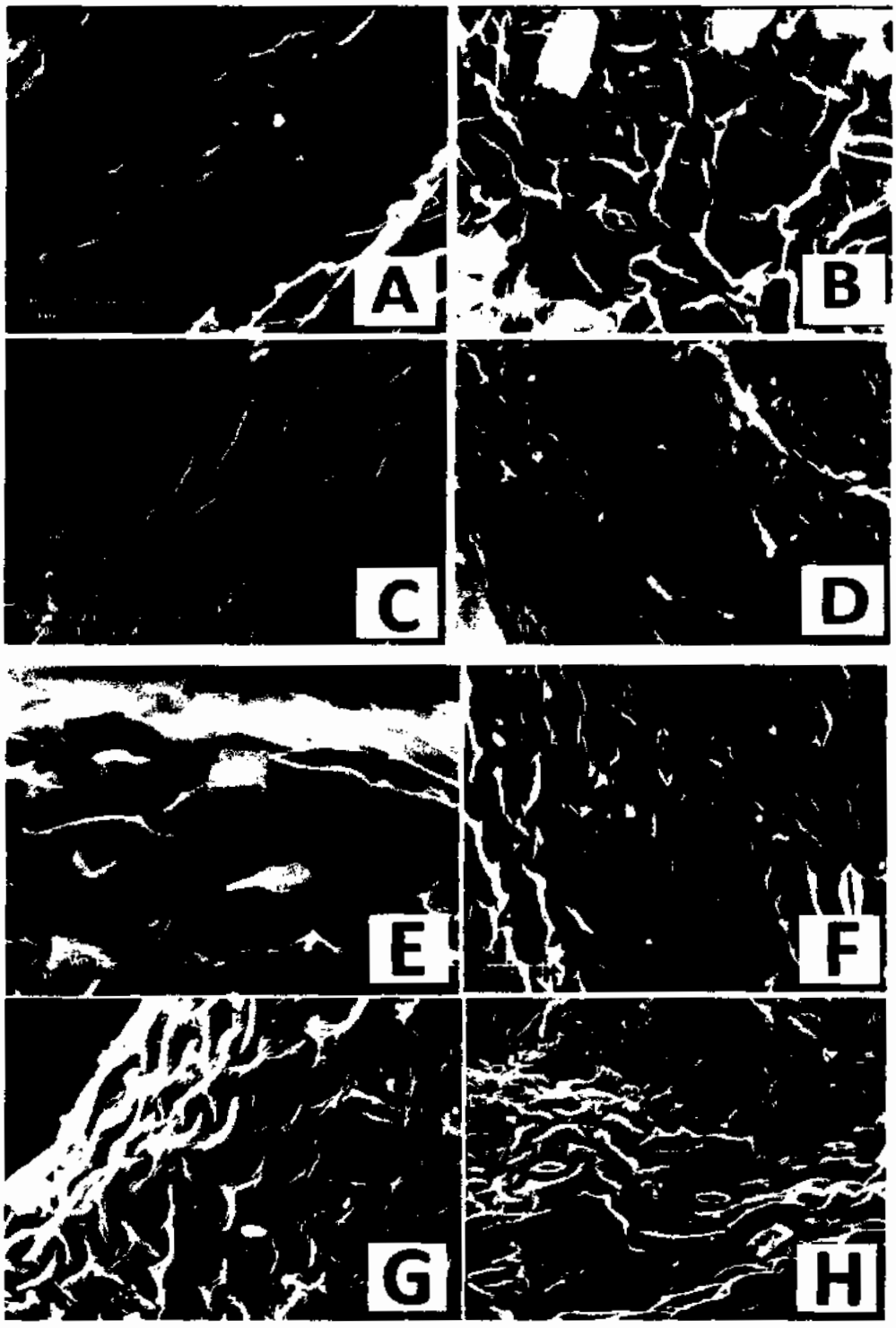

Plate 4.37. Scanning Electron micrographs of foliar epidermal cells of different *Artemisia* species: (A) *A. annua* (B) *A. argyi* (C) *A.* sp. AD-H (D) *A. maritima* (E) *A. rutifolia* (F) *A. campestris* (G) *A. chamaemelifolia* (H) *A. tournefortiana.* Scale bar = 2, 10 & 100 µm.

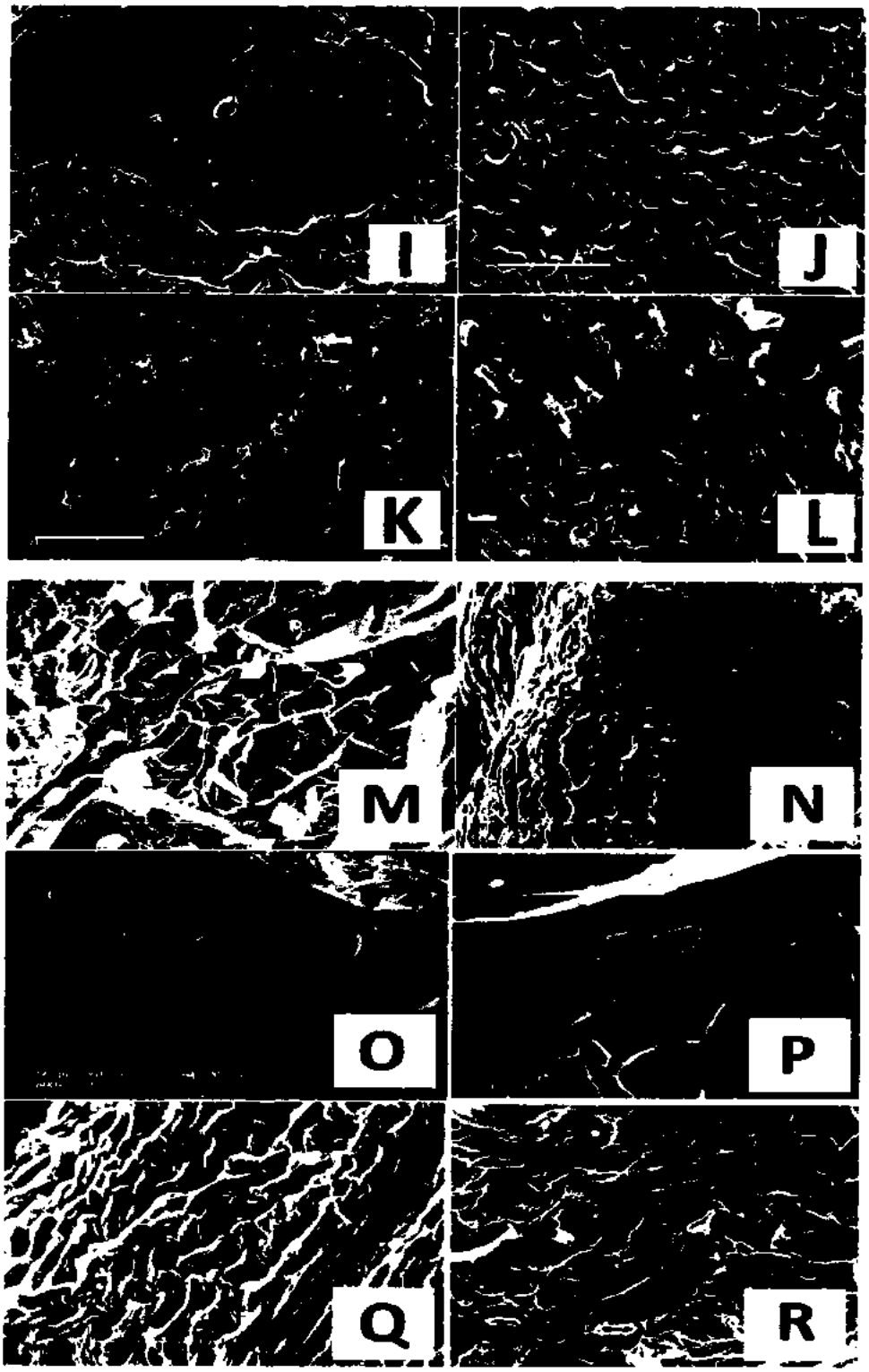

Plate 4.37. Continued... (I) *A.verlotiorum* (J) *A. indica* (K) *A. scoparia* (L) *A. chinensis* (M) *A. austriaca* (N) *A. gmelinii* (O) *A. herba-alba* (P) *A. pontica* (Q) *A. vulgaris* (R) *A. montana*. Scale bar = 2, 10 & 100 μm.

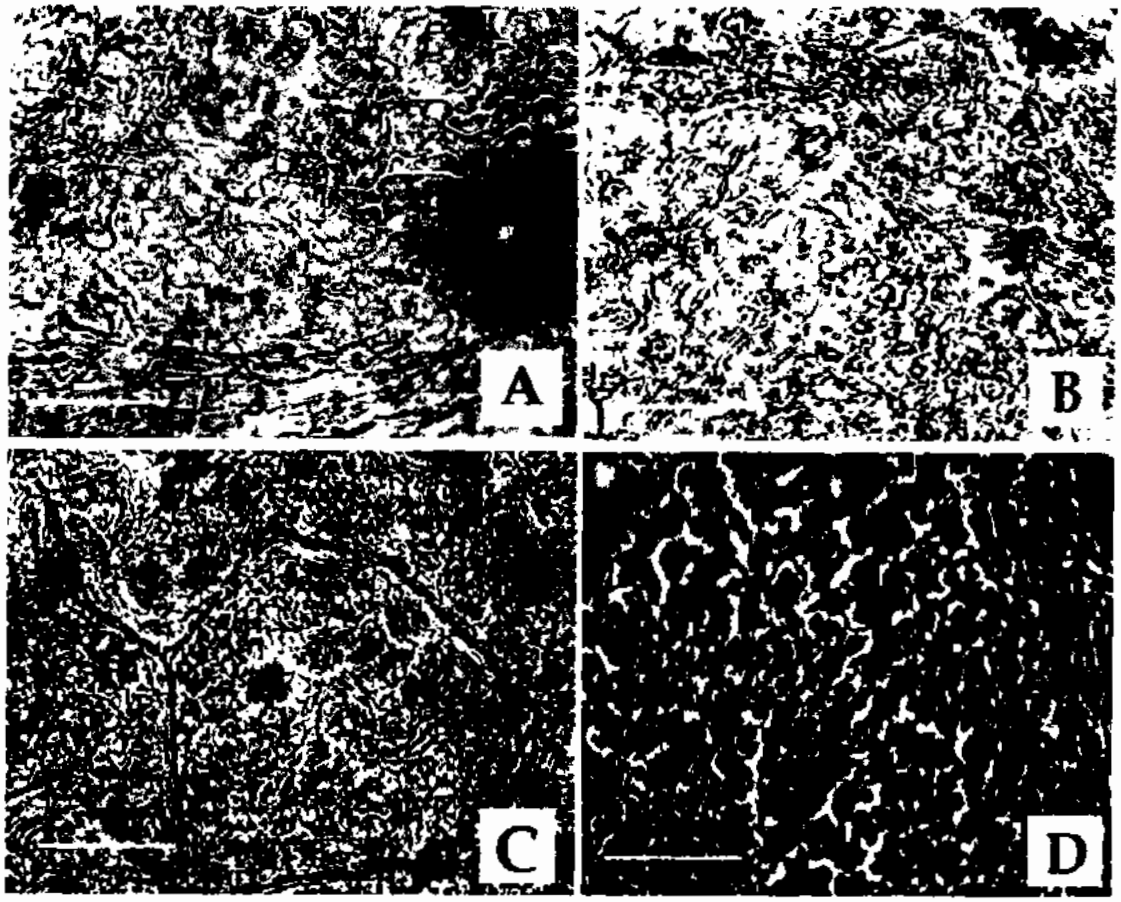

Plate 4.38. Epidermal cells arrangement in *Artemisia* by means of LM: (A) *A. annua* (B) *A. chinensis* (C) *A. dubia* (D) *A. indica* (Scale bar = 50-100µm).

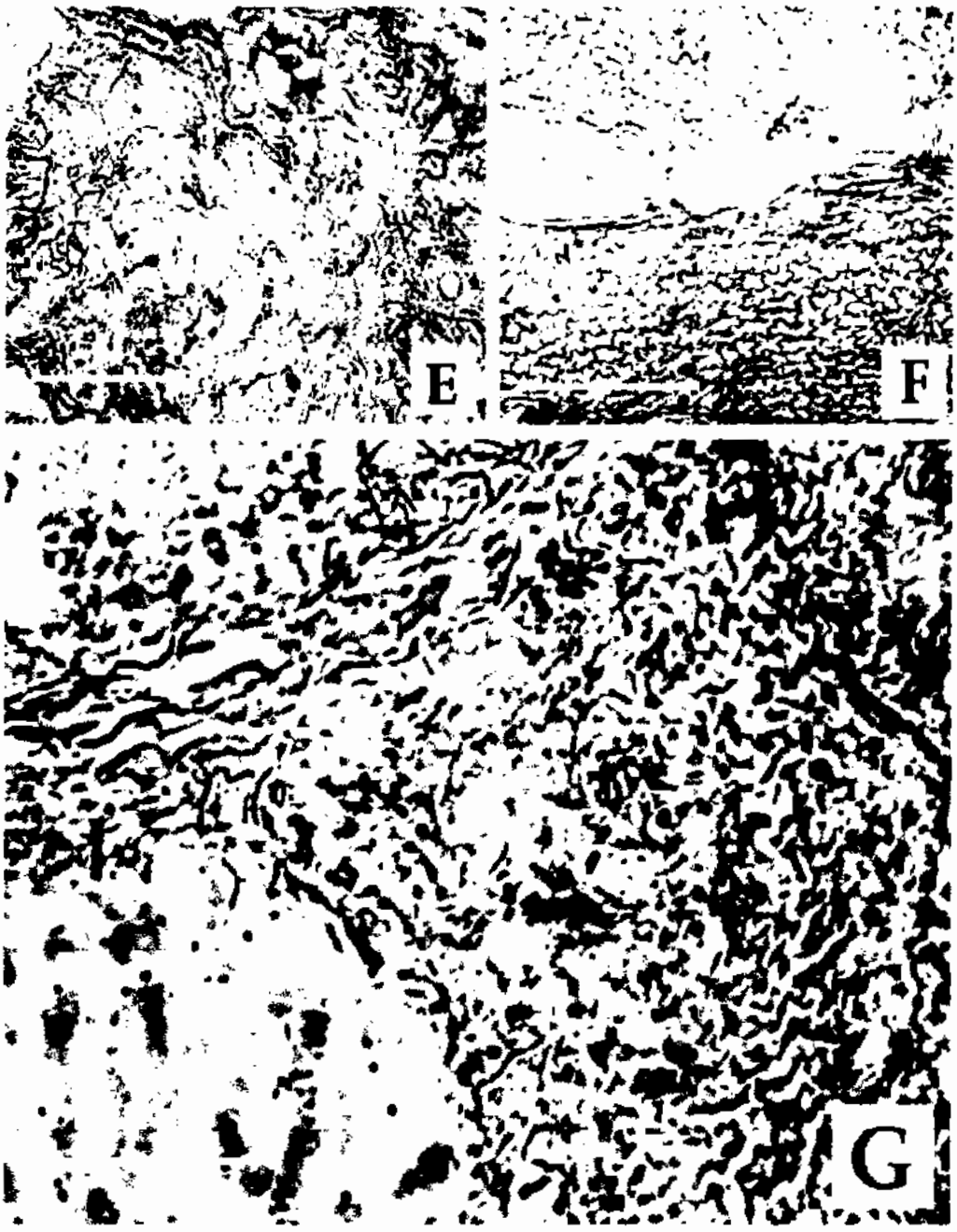

Plate 4.38. Continued... (A) *A. annua* (B) *A. chinensis* (C) *A. dubia* (D) *A. indica* (E) *A. tournefortiana* (F) *A. verlotiorum* (G) *A. vulgaris*. Scale bar = 50-100µm.

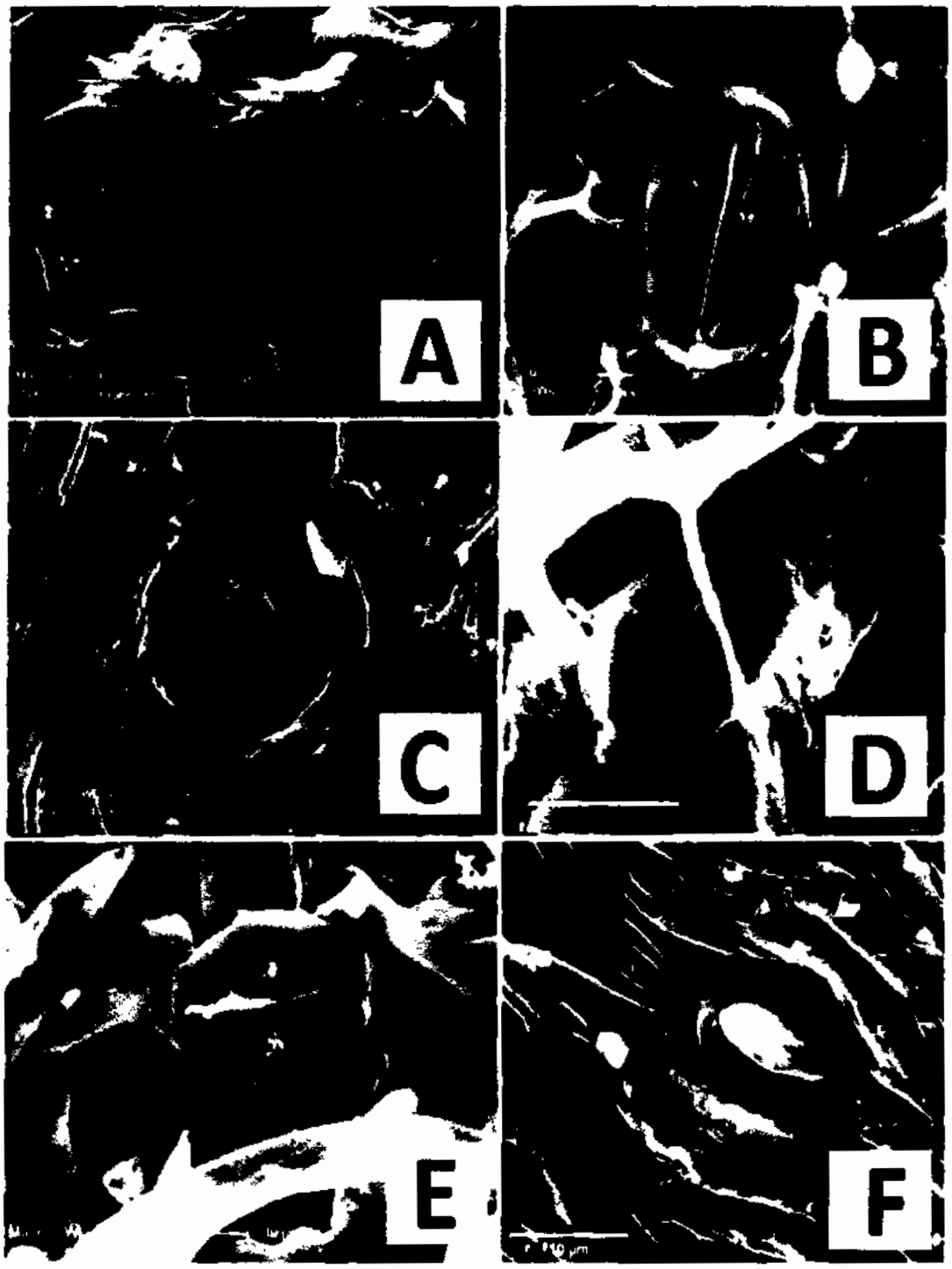

Plate 4.39. Scanning Electron micrographs showing stomatal variations in different *Artemisia* species: (A) *A. annua* (B) *A. argyi* (C) *A.* sp. AD-H (D) *A. maritima* (E) *A.herba-alba* (F) *A. campestris.* Scale bar = 10 µm.

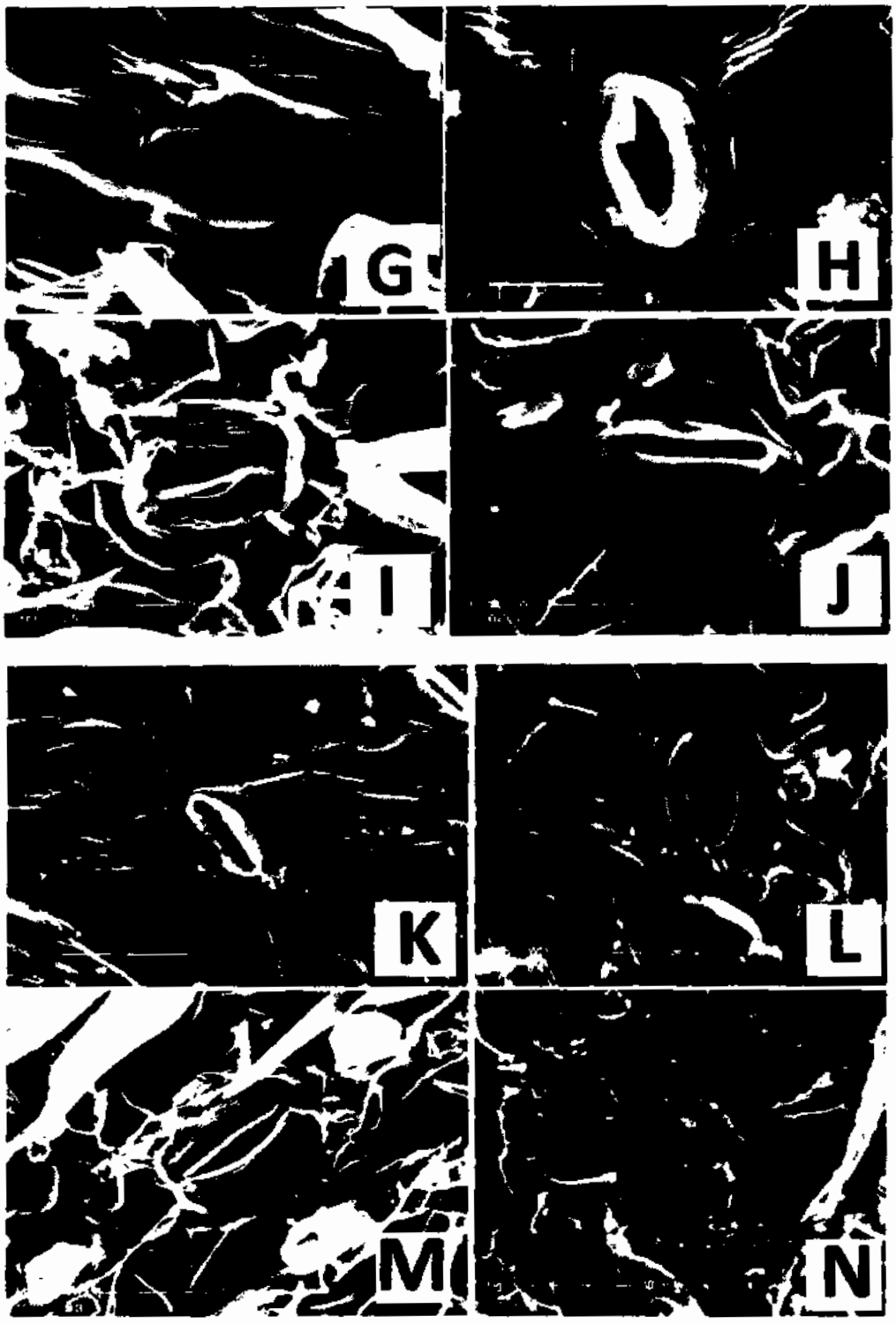

Plate 4.39. Continued... (G) *A. chamaemelifolia* (H) *A. tournefortiana* (I) *A. verlotiorum* (J) *A. indica* (K) *A. scoparia* (L) *A. chinensis* (M) *A. austriaca* (N) *A. gmelinii*. Scale bar = 10 μm.

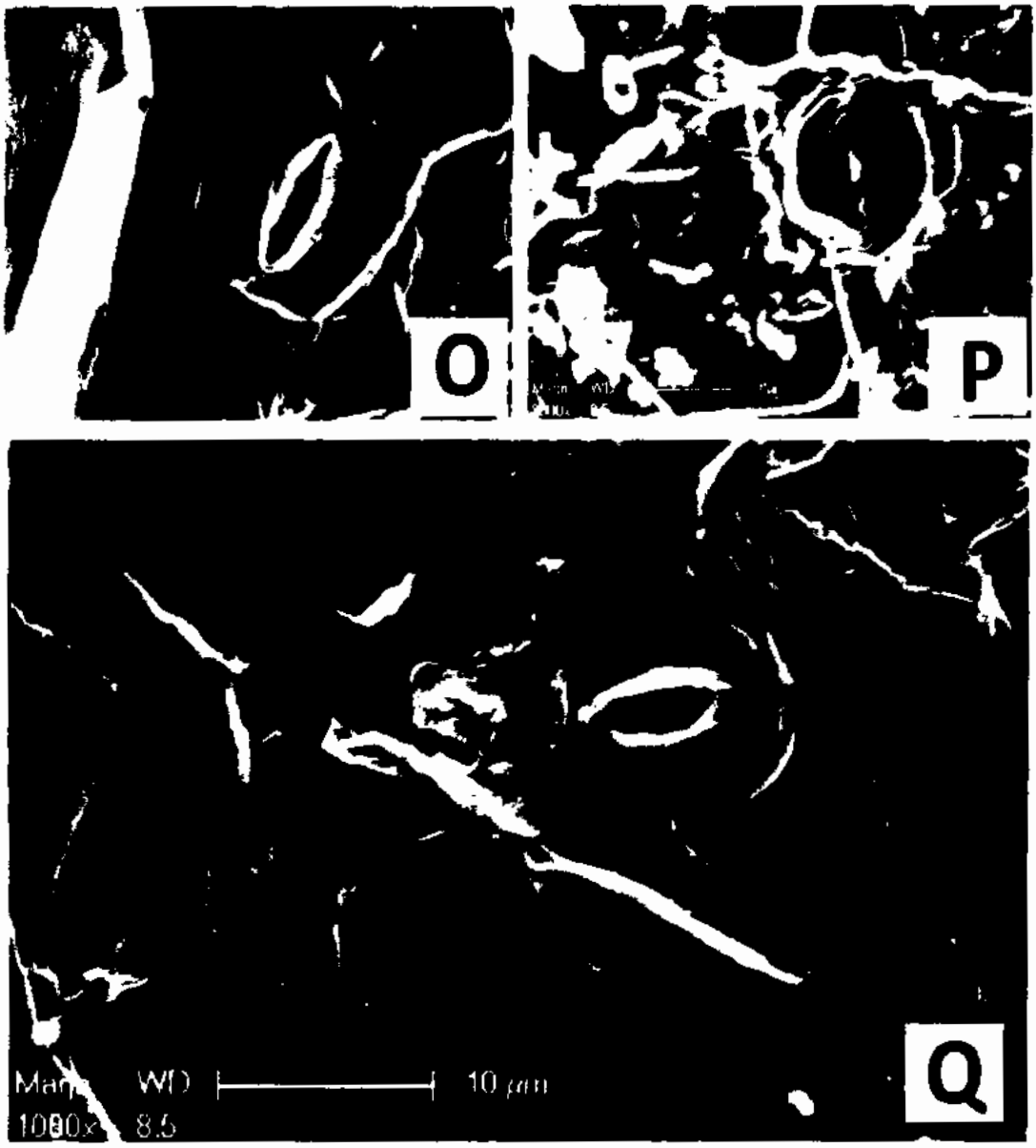

Plate 4.39. Continued... (O) *A. pontica* (P) *A. vulgaris* (Q) *A. montana*. Scale bar = 10 µm.

4.3.2. Foliar Trichomes

In this study, the distribution of foliar trichomes in 17 *Artemisia* species obtained from Gilgit-Baltistan region of Pakistan were assessed and presented in the form of plates. A total of 10 main types of trichomes (including glandular and non-glandular) were noticed using light and scanning electron microscopic observations (Plate 4.40, 4.41 and 4.42). The glandular trichomes were of 4 types including peltate, pluricellular, capitate and thin necked trichomes, while non-glandular trichomes were of 6 types including, Aduncate, unicellular calavate, conical type, stinging hair type, unicellular tector and unicellurar filiform.

The quantitative attributes of glandular and non-glandular foliar trichomes in *Artemisia* species are given in Table 4.5 and 4.6.

The details of all the investigated trichomes are given below.

(I). Capitate trichomes. These glandular trichomes have ellipsoid like appearance. In this study we found these types of trichomes in *A. argyi* (Plate 4.40-B), *A. dubia* (Plate 4.42-C), *A. tournefortiana* (Plate 4.40-D), *A. verlotiorum* (Plate 4.40-F), *A. vulgaris* (Plate 4.40-N), *A.* sp.-A (Plate 4.40-E), *A.* sp.-H. (Plate 4.40-I) and *A.* sp.-I (Plate 4.40-M).

(II). Peltate tricomes. These trichomes are glandular, looks like a ball with multicellular structures and are common in *A. austriaca* (Plate 4.40-H), *A. chinensis* (Plate 4.40-K, Figure 4.42-B), *A. indica* (Plate 4.42-D) and *A. montana* (Plate 4.40-J)

(III). Pluricellular trichomes. These glandular trichomes are long with 2 to 5 cells. They look broader from the base and tapering towards the top. These type of trichomes are found in *A. annua* (Plate 4.40-A, Plate 4.42-E), *A. chamaemelifolia* (Plate 4.40-C, Plate 4.42-A) and *A.* sp.-G (Plate 4.40-G).

(IV). Thin neck trichomes. They have short neck with large head and these glandular trichomes are found in *A. gmelinii* (Plate 4.40-L).

(V). Conical type trichomes. These are non-glandular trichomes present in conical shape having a circular base tapering towards the apex. this study found these types of trichomes in *A. chinensis* (Plate 4.41-G) and *A.* sp.-A (Plate 4.41-D).

(VI). Stinging hair trichomes. These trichomes are sharp with a sting like apex. These trichomes are non-glandular and are found in *A. argyi* (Plate 4.41-A).

(VII). Aduncate. These types of trichomes are non-glandular. They are long with curved or hook like apex and are present in *A. dubia* (Plate 4.42-G), *A. indica* (Plate 4.41-F, Figure 4.42-F), *A. verlotiorum* (Plate 4.41-E) and *A.* sp.-I (Plate 4.41-B)

(VIII). Unicellular calavate. These trichomes are also non-glandular. They are short and narrow from the base and thicker at the apex. These types of trichomes were seen in *A. tournefortiana* (Plate 4.41-C)

(IX). Unicellular filiform. These trichomes are sharper at the apex. These trichomes are non glandular and are found in *A. austriaca* (Plate 4.41-H) and *A.* sp.-H (Plate 4.41-I).

(X). Unicellular tector. They are non-glandular. They are present in clusters or thread like. These types of trichomes are found in *A. herba-alba* (Plate 4.41-J).

Table 4.5. Quantitative attributes of glandular foliar trichomes in *Artemisia* species.

Artemisia spp.	Capitate trichome		Peltate trichome		Pluricellular trichome		Thin neck trichome	
	Presence/ Absence	Height x width (µm)	Presence/ Absence	Diameter (µm)	Presence /Absence	Height x width (µm)	Presence /Absence	Height x width (µm)
A. annua	Absent	—	Absent	—	Present	50.02-52.06x17.58-19.20	Absent	—
A. argyi	Present	44.19-46.49x31.60-31.87	Absent	—	Absent	—	Absent	—
A. austriaca	Absent	—	Present	81.38-83.85	Absent	—	Absent	—
A chinensis	Absent	—	Present	61.22-63.72	Absent	—	Absent	—
A. chamemelifolia	Absent	—	Absent	—	Present	48.12-50.30x18.57-24.37	Absent	—
A. dubia	Present	—	Absent	—	Absent	—	Absent	—
A.gmelinii	Absent	—	Absent	—	Absent	—	Present	41.81-42.26x8.09-10.15
A. indica	Absent	—	Present	—	Absent	—	Absent	—
A.montana	Absent	—	Present	34.13-40.31	Absent	—	Absent	—
A. tournefortiana	Present	39.84-41.20x24.14-27.74	Absent	—	Absent	—	Absent	—
A. verlotiorum	Present	47.30-49.56x36.28-38.31	Absent	—	Absent	—	Absent	—
A. vulgaris	Present	65.24-66.30x35.52-41.97	Absent	—	Absent	—	Absent	—
A. sp. - A	Present	60.70-63.86x38.30-39.65	Absent	—	Absent	—	Absent	—
A. sp. -G	Absent	—	Absent	—	Present	29.69-31.30x10.13-16.62	Absent	—
A. sp. - H	Present	38.43-45.17x28.46-29.67	Absent	—	Absent	—	Absent	—
A. sp. - I	Present	65.24-66.30x35.52-41.97	Absent	—	Absent	—	Absent	—

Table 4.6. Quantitative attributes of non-glandular foliar trichomes in *Artemisia* species.

Artemisia spp.	**Aduncate**		**Unicellular Clavate**		**Conical trichome**	
	Presence/ Absence	**Height x width (µm)**	**Presence/ Absence**	**Lengthx width (µm)**	**Presence/ Absence**	**Height x width (µm)**
A. austriaca	Absent	—	Absent	—	Absent	
A. argyi	Absent	—	Absent	—	Absent	—
A. chinensis	Absent	—	Absent	—	Present	47.95-50.51x15.21-23.76
A. dubia	Present	—	Absent	—	Absent	—
A. herba-alba	Absent	—	Absent	—	Absent	—
A. indica	Present	189.55-199.30x7.87-10.90	Absent	—	Absent	—
A. tournefortiana	Absent	—	Present	68.80-70.65x16.70-21.12	Absent	—
A. verlotiorum	Present	130.46-127.30x9.94-12.64	Absent	—	Absent	—
*A.*sp. -A	Absent	—	Absent	—	Present	106.41-108.32x6.65-23.46
A. sp. -H	Absent	—	Absent	—	Absent	—
A. sp. -I	Present	120.46-124.30x11.94-14.64	Absent	—	Absent	—

Table 4.6. Continued…

Artemisia spp.	Stinging hair trichome		Unicellular filiform		Unicellular tector	
	Presence /Absence	Height x width (µm)	Presence/ Absence	Height x width (µm)	Presence/ Absence	Height x width (µm)
A. austriaca	Absent	---	Present	133.89-135.30x7.64-12.09	Absent	---
A. argyi	Present	159.03-161.30x11.40-13.24	Absent	---	Absent	---
A. chinensis	Absent	---	Absent	---	Absent	---
A. herba-alba	Absent	---	Absent	---	Present	90.11-94.20x2.30-3.25
A. indica	Absent	---	Absent	---	Absent	---
A. tournefortiana	Absent	---	Absent	---	Absent	---
A. verlotiorum	Absent	---	Absent	---	Absent	---
A. sp. -A	Absent	---	Absent	---	Absent	---
A. sp. -H	Absent	---	Present	285.11-289.94x13.79-14.52	Absent	---
A. sp. -I	Absent	---	Absent	---	Absent	---

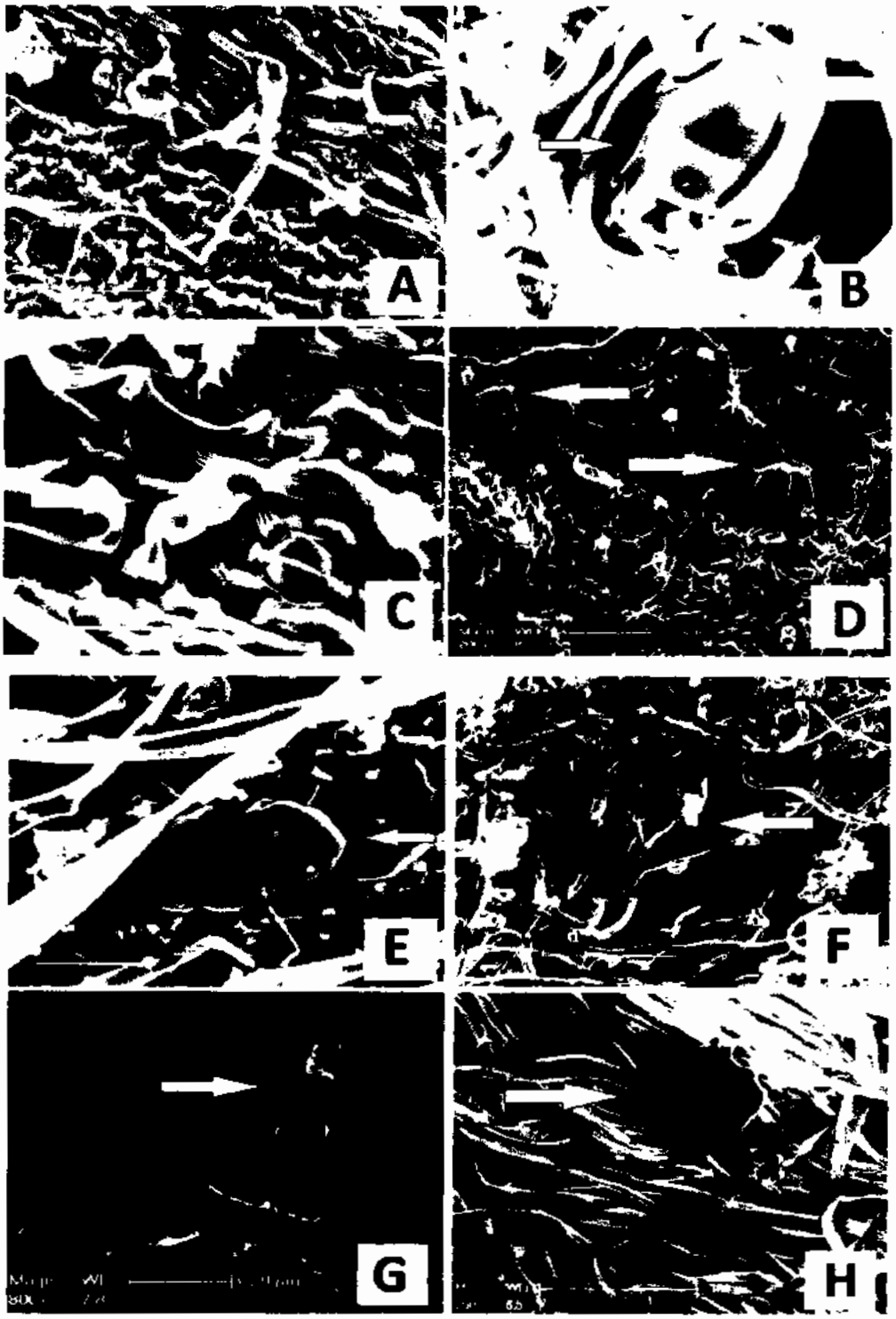

Plate 4.40. Scanning electron micrographs showing glandular trichomes of different *Artemisia* species: (A) *A. annua* (B) *A.argyi* (C) *A. chamaemelifolia* (D) *A. tournefortiana* (E) *A.* sp. –A (F) *A. verlotiorum* (G) *A.* sp. –G (H) *A. austriaca*. Scale bar = 20-200 µm.

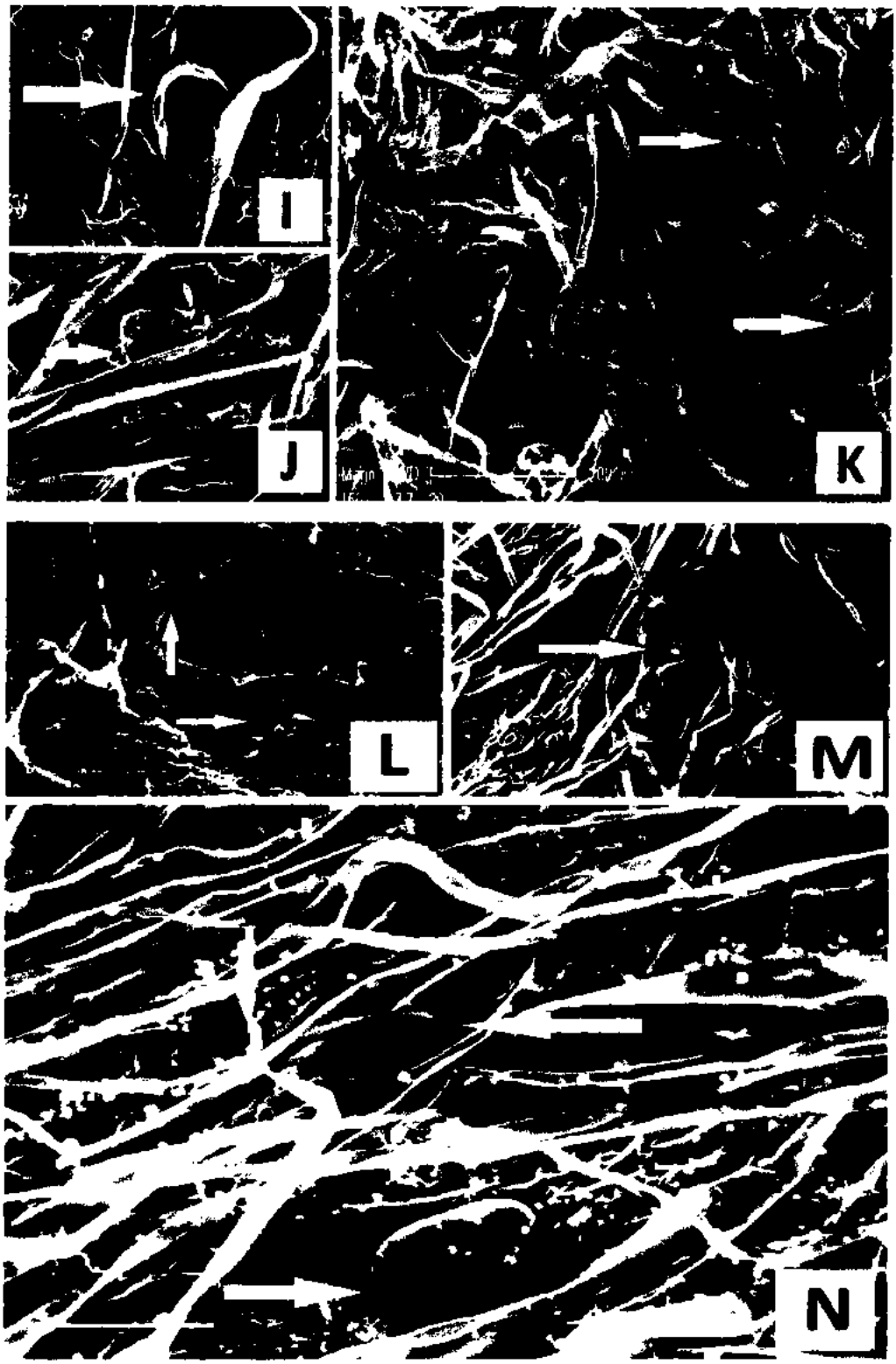

Plate 4.40. Continued... (I) *A.* sp. -H (J) *A. montana* (K) *A. chinensis* (L) *A. gmelinii* (M) *A.* sp.-I (N) *A. vulgaris.* Scale bar = 20-200 µm.

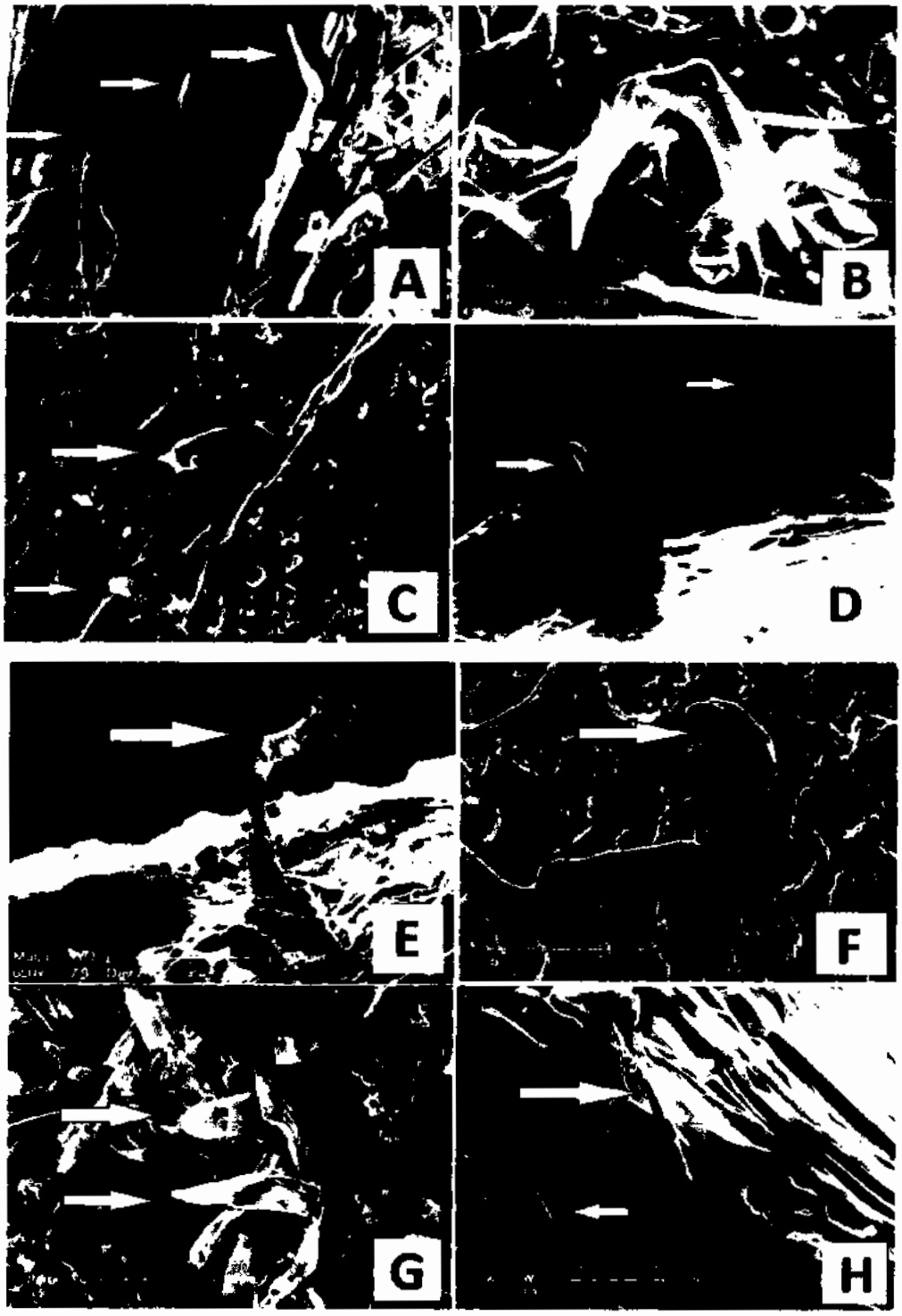

Plate 4.41. Scanning electron micrographs showing non glandular trichomes of different *Artemisia* species: (A) *A. argyi* (B) *A.* sp. –I (C) *A. tournefortiana* (D) *A.* sp. –A (E) *A. verlotiorum* (F) *A. indica* (G) *A. chinensis* (H) *A. austriaca*. Scale bar = 20-100 µm.

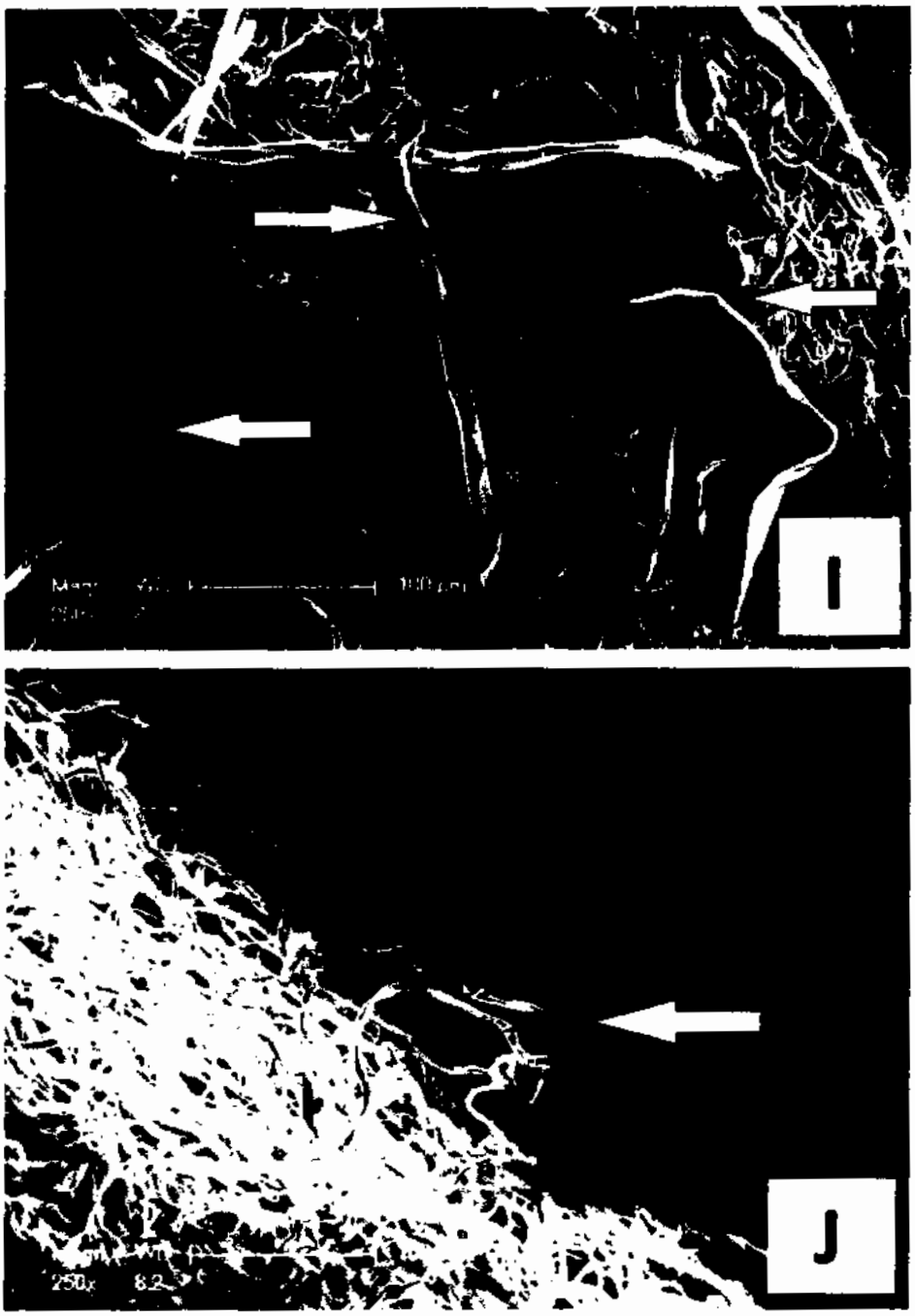

Plate 4.41. Continued... (I) *A.* sp. –H (J) *A. herba-alba.* Scale bar = 20-100 µm.

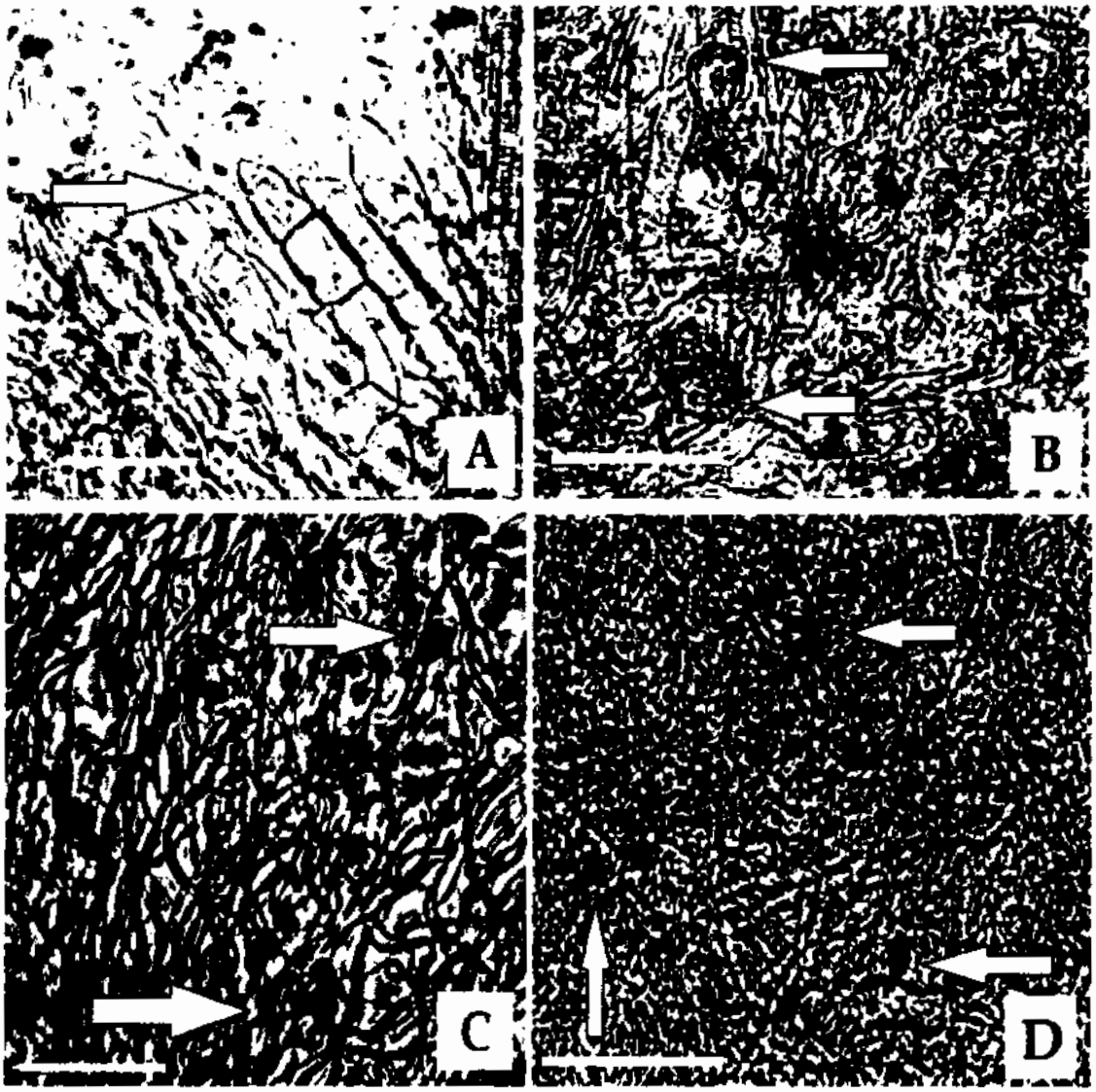

Plate 4.42. LM monographs of trichomes of different *Artemisia* species: (A) *A. chamaemelifolia* (B) *A. chinensis* (C) *A. dubia* (D) *A. indica.* (Scale bar = 50-100 µm).

Plate 4.42. Continued... (E) *A. annua* (F) *A. indica* (G) *A. dubia*. Scale bar = 50-100 µm.

4.4. Pollen morphology

This study found some variation in the pollen structure of different *Artemisia* species. The characteristics of pollen of different *Artemisia* species investigated in this study includes equatorial (E) measurements, polar view (P), Polar and equatorial ratio, pollen shape, spinules presence/absence and the ornamentation of exine.

All pollen structures and measurements for the examined species are given in Table 4.7 and also illustrated in Plate 4.43, 4.44 and 4.45. The equatorial and polar views of the *Artemisia* pollen are presented in plate 4.43 and 4.44. The patrons of sculpture of exine surface of *Artemisia* pollen are shown in Plate 4.45, where the presence of tiny spinules can also be seen. Similarly the spinules densities recorded showed variation among different species of *Artemisia*. These spinules on the other hand are very unique in all investigated *Artemisia* species. Few investigated species showed some progressive tendency in their structure.

The phylogenetic tree based on micro morphological traits of pollen is given in Figure 4.15. From the light microscopic and scanning electron microscopic observations, the shape of pollen grain was found to be clearly homogeneous throughout in the genus and authenticates its monophyly.

The general features of *Artemisia* pollen are recognized by the the approximate symmetry or globular 3 lobed spheres from equatorial side whereas ellipsoid from the polar side. By LM and SEM, total 7 characters of *Artemisia* pollen were selected for cladistics and cluster analysis using the PHYLIP and UPGMA softwares (Table 3.3).

A data matrix was generated using these micro morphological traits of pollen for cladistics and cluster analysis (Table 4.8). From the MPTs, a strict consensus cladogram (Figure 4.15) obtained on the basis of micromorphological traits of pollen of *Artemisia* shows the origin of different lineages of *Artemisia* at different points. This cladistic analysis also indicates the comparison with traditional classification.

In the resulting cluster analysis, 5 groups within *Artemisia* have been recognized. *A. chinensis, A. chamaemelifolia* and *A. tournefortiana* falls in group 1, while *A.* sp. -A form group 2. In the group 3, *A. gmelinii, A. herba-alba, A. indica, A. pontica, A. sp.* -B and *A.* sp. -H were found.

In the resulting analysis, the group 4 includes species like *A. austriaca*, *A. campestris*, *A. montana*, *A. vulgaris*, *A.* sp.-F, *A.* sp.-G, and *A.* sp.-I. In the 5th group, *Artemisia* species like *A. annua*, *A. maritima*, *A. rutifolia*, *A. scoparia*, and *A.* sp.-D were included as shown in Figure 4.16.

Table 4.7. Quantitative characteristics of pollen of different *Artemisia* species.

***Artemisia* spp.**	**Polar (µm)**	**Equatorial (µm)**	**P/E Sphericity**
A. annua	15.60	18.79	0.83
A. austriaca	17.20	16.49	1.04
A. campestris	15.55	15.75	0.98
A. chamaemelifolia	20.30	19.04	1.06
A. chinensis	24.24	12.01	2.01
A. gmelinii	16.86	14.90	1.13
A. herba-alba	22.58	18.64	1.21
A. indica	17.65	14.37	1.22
A. maritima	15.59	14.49	1.07
A. montana	15.45	16.54	0.93
A. pontica	17.86	14.65	1.21
A. rutifolia	16.70	17.43	0.95
A. scoparia	15.12	17.36	0.87
A. tournefortiana	19.08	19.59	0.97
A. vulgaris	15.33	16.93	0.90
A. sp. -A	20.26	18.40	1.10
A. sp. -B	15.83	14.20	1.11
A. sp. -D	15.85	18.06	0.87
A. sp. -F	16.98	15.20	1.11
A. sp. -G	16.71	15.32	1.09
A. sp. -H	15.26	14.44	1.05
A. sp. -I	17.62	16.12	1.09

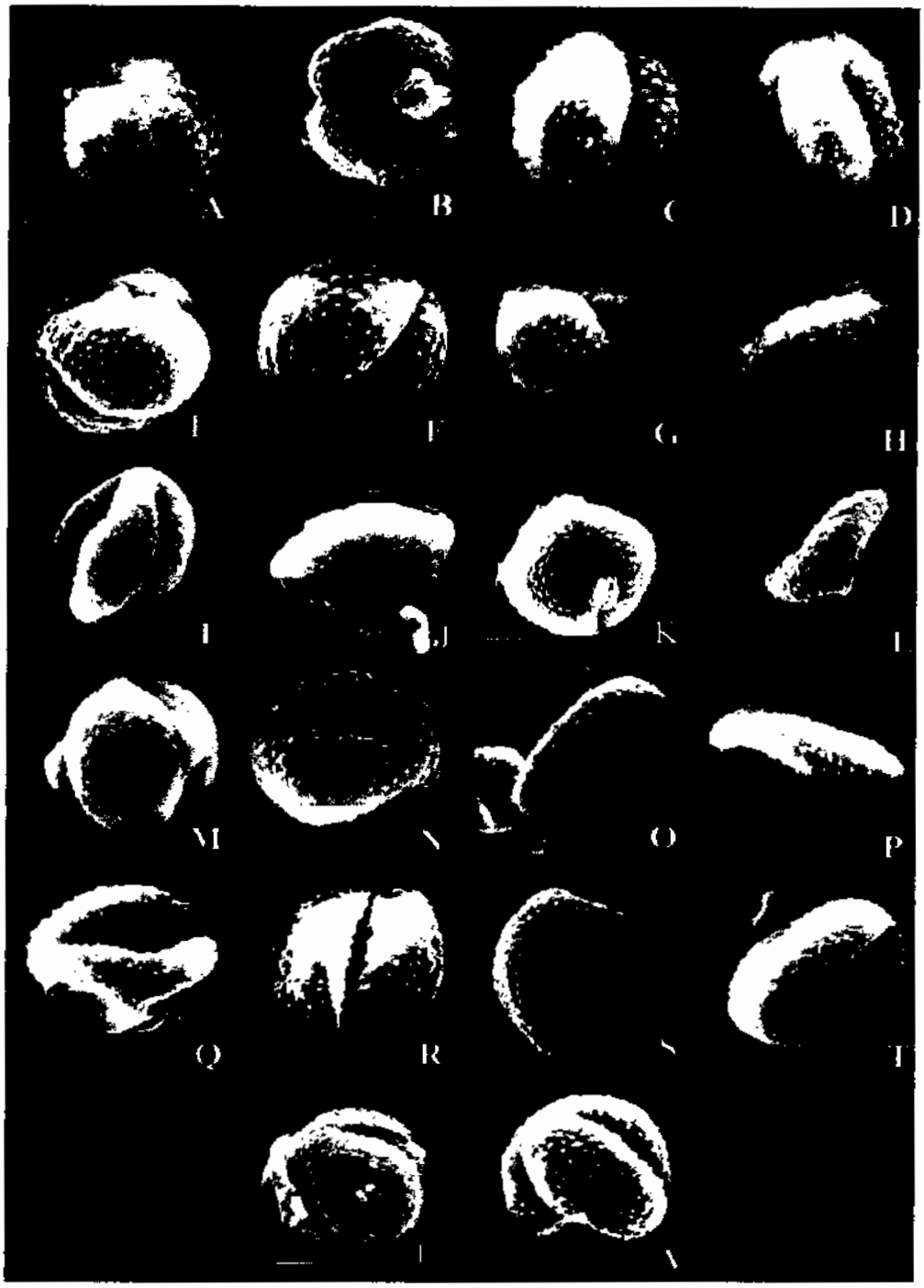

Plate 4.43. Scanning electron micrographs showing the equatorial view of pollens of *Artemisia* species: (A) *A. annua* (B) *A. maritima* (C) *A.* sp.-I (D) *A.* sp.-F (E) *A. rutifolia* (F) *A. campestris* (G) *A. chamaemelifolia* (H) *A. tournefortiana* (I) *A.* sp.-A (J) *A.* sp.-B (K) *A.* sp.-D (L) *A. indica* (M) *A.* sp.-G (N) *A. scoparia* (O) *A. chinensis* (P) *A. austriaca* (Q) *A. gmelinii* (R) *A.* sp.-H (S) *A. herba-alba* (T) *A. pontica* (U) *A. vulgaris* (V) *A. montana.* Scale bar = 2-10µm.

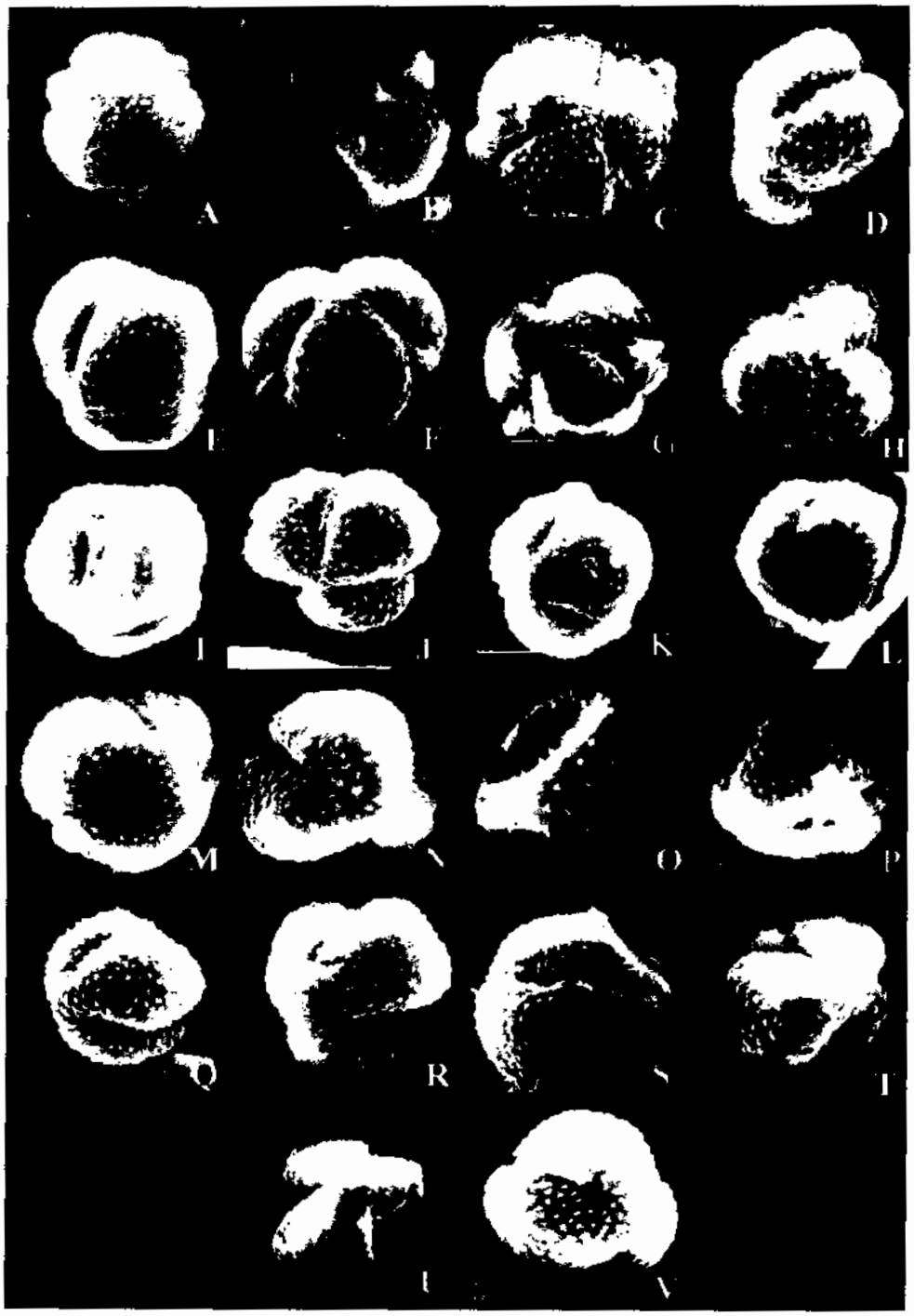

Plate 4.44. Scanning electron micrographs showing the polar view of pollens of *Artemisia* species: (A) *A. annua* (B) *A. maritima* (C) *A.* sp.-I (D) *A.* sp.-F (E) *A. rutifolia* (F) *A. campestris* (G) *A. chamaemelifolia* (H) *A. tournefortiana* (I) *A.* sp.-A (J) *A.* sp.-B (K) *A.* sp.-D (L) *A. indica* (M) *A.* sp.-G (N) *A. scoparia* (O) *A. chinensis* (P) *A. austriaca* (Q) *A. gmelinii* (R) *A.* sp.-H (S) *A. herba-alba* (T) *A. pontica* (U) *A. vulgaris* L (V) *A. montana.* Scale bar = 2-10µm.

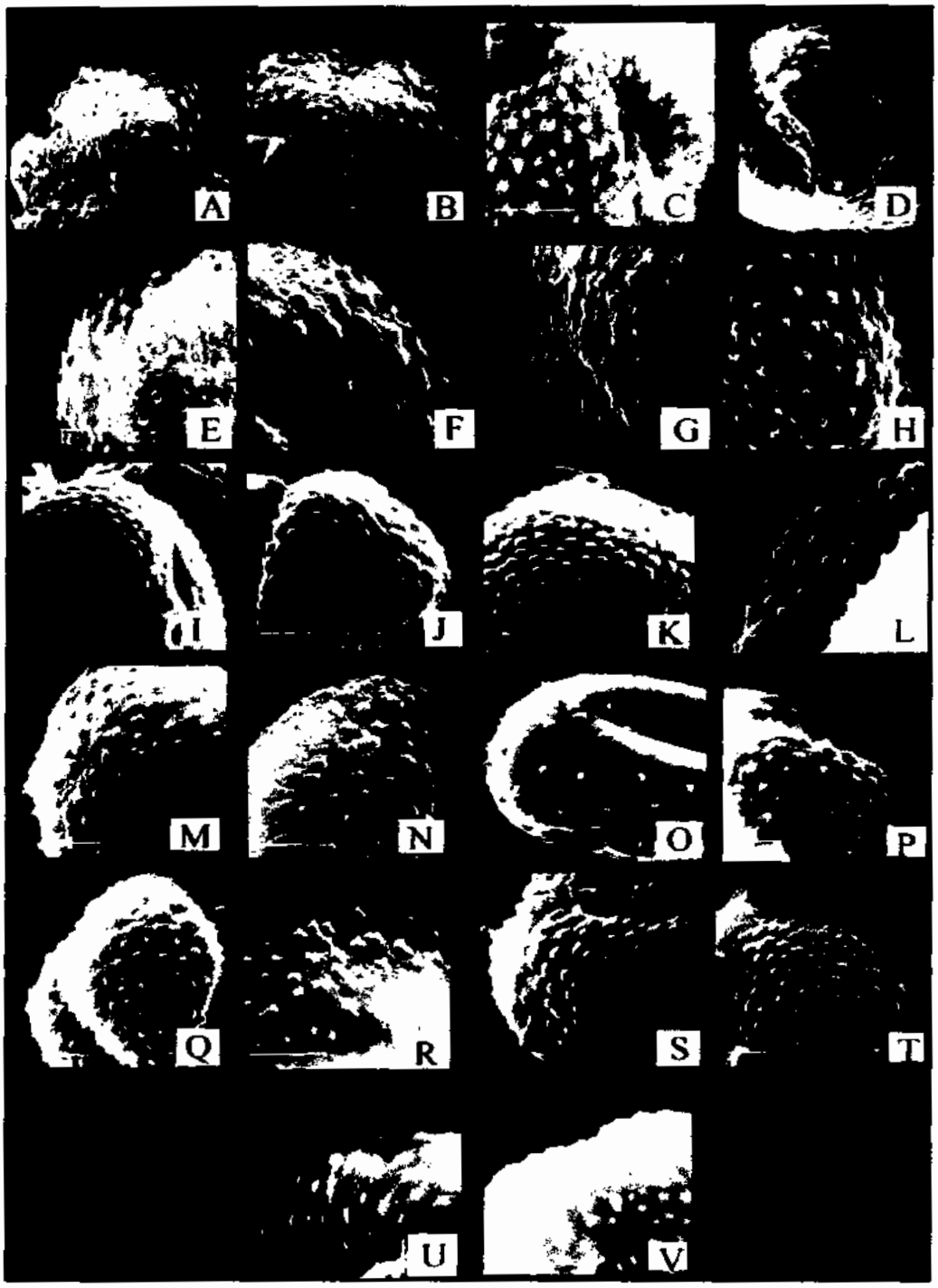

Plate 4.45. Scanning electron micrographs showing the exine sculpture view of pollens of *Artemisia* species: (A) *A. annua* (B) *A. maritima* (C) *A.* sp.-I (D) *A.* sp-F (E) *A. rutifolia* (F) *A. campestris* (G) *A. chamaemelifolia* (H) *A. tournefortiana* (I) *A.* sp.-A (J) *A.* sp.-B (K) *A.* sp.-D (L) *A. indica* (M) *A.* sp.-G (N) *A. scoparia* (O) *A. chinensis* (P) *A. austriaca* (Q) *A. gmelinii* (R) *A.* sp.-H (S) *A. herba-alba* (T) *A. pontica* (U) *A. vulgaris* (V) *A. montana.* Scale bar = 2-5µm.

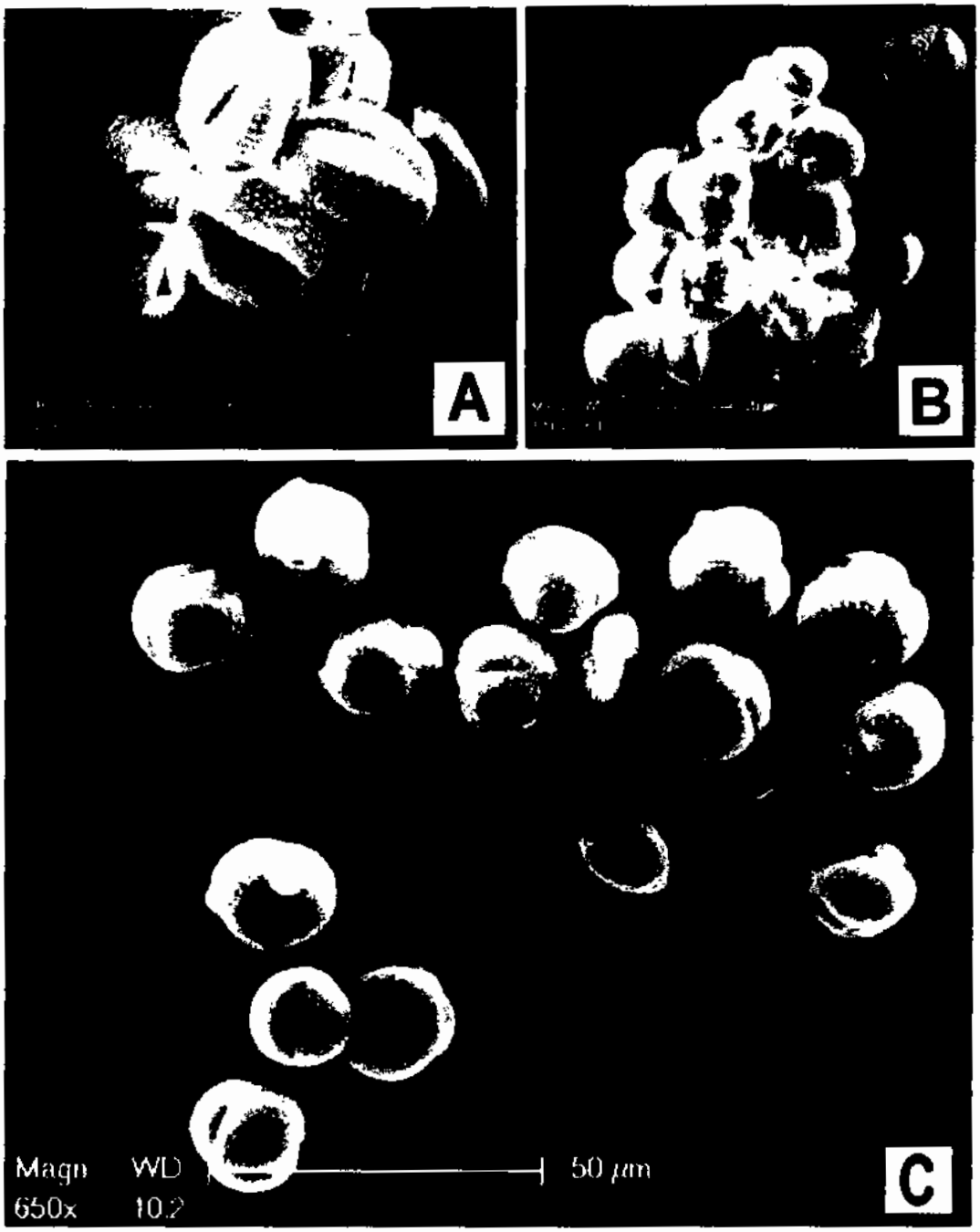

Plate 4.46. Scanning electron micrographs showing pollens ornamentation in *Artemisia* species: (A) *A. pontica* (B) *A. vulgaris* (C) *A. rutifolia.* Scale bar = 10, 20 and 50µm.

Table 4.8. Character state matrix for cluster analysis of different *Artemisia* species based on morphological features of pollen. Characters and character states are already presented in Table 3.3.

S/No	***Artemisia* spp.**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	*A. annua*	1	1	0	1	1	9	3
2	*A. austriaca*	1	1	1	0	0	7	5
3	*A. campestris*	1	0	1	1	0	9	6
4	*A. chamaemelifolia*	1	1	0	1	1	4	2
5	*A. chinensis*	1	1	1	0	1	1	1
6	*A. gmelinii*	1	1	0	1	1	8	7
7	*A. herba-alba*	1	1	1	0	1	9	7
8	*A. indica*	1	1	1	1	1	7	7
9	*A. maritima*	1	1	1	0	1	9	3
10	*A. montana*	1	0	0	0	1	9	5
11	*A. pontica*	1	1	0	0	1	7	7
12	*A. rutifolia*	1	0	1	0	0	8	4
13	*A. scoparia*	1	0	1	1	0	9	4
14	*A. tournefortiana*	1	1	1	1	1	5	2
15	*A. vulgaris*	1	0	0	1	0	9	5
16	*A.* sp.- A	1	1	0	0	1	4	6
17	*A.* sp.- B	1	1	0	0	1	9	7
18	*A.* sp.- D	1	0	0	0	1	9	3
19	*A.* sp.- F	1	1	1	0	0	8	6
20	*A.* sp.- G	1	0	1	1	1	8	6
21	*A.* sp.- H	1	1	1	0	0	9	7
22	*A.* sp.- I	1	1	0	0	1	7	5
	OUT	0	0	0	0	0	0	0

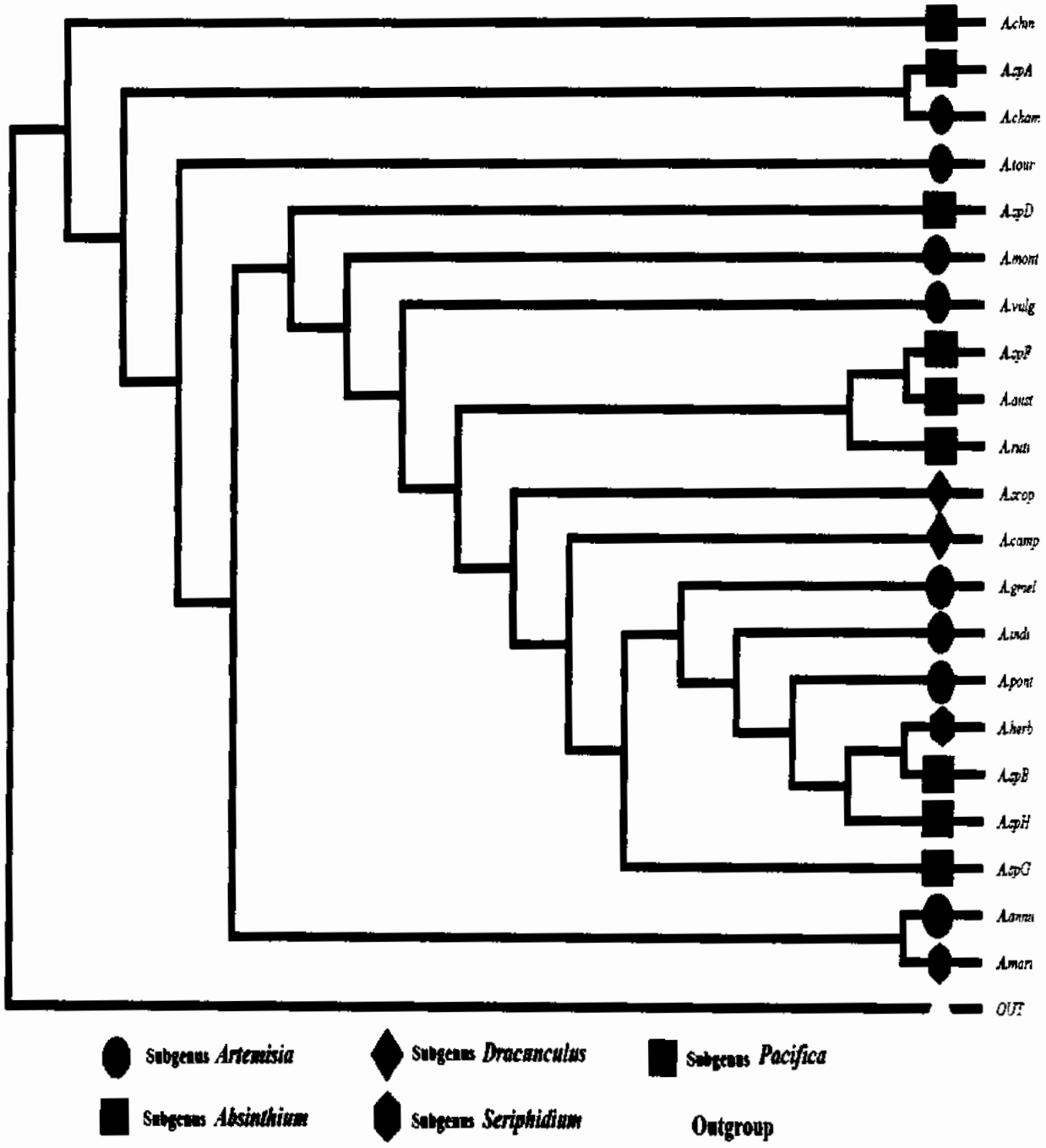

Figure 4.15. The strict consensus cladogram of *Artemisia* based on the micromorphological characters of pollen grains. The tree was rooted with an imaginary outgroup. Colored geometrical shapes indicate the traditional subgeneric classification of *Artemisia* based on floral morphology.

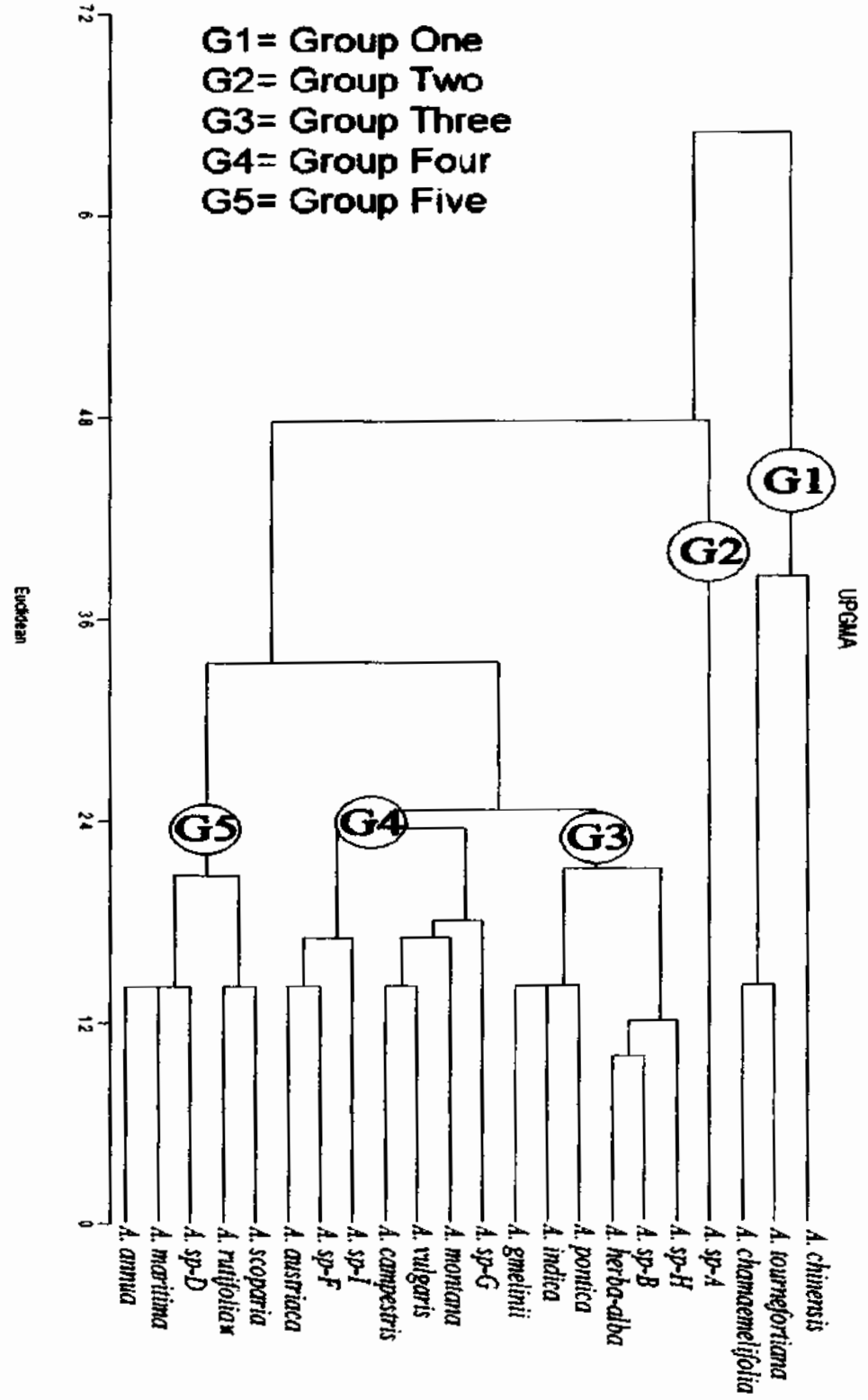

Figure 4.16. Dendrogram based on cluster analysis of pollen micromorphological characters of different species of genus *Artemisia*.

4.5. Phytogeography

Figure 3.1 represent the localities of *Artemisia* diversity in Gilgit-Baltistan region of Pakistan. *Artemisia* species are mostly distributed in all parts of Gilgit-Baltistan covering the west Himalayan slopes. It was observed that high altitude arias with moderate precipitation serve as home to indigenous *Artemisia* species. *Artemisia* species mostly founded on sun facing slops of the mountains.

It was observed that irrespective of environmental conditions required for the growth of genus *Artemisia*. *A. sieversiana* and *A. maritima* were distributed in all phytogeographical regions of Gilgit-Baltistan region.

A. maritima and *A. herba-alba* covers most of the mountains as dominant species in the Gilgit-Baltistan region of Pakistan, especially Gilgit and Ghizer valleys as shown in plate 4.47 and 4.48. Naltar region was identified as hot spot area for species diversity.

A.indica and *A. dubia* was distributed in Ghizer and Gilgit area while *A. rutifolia* and *A. chamaemelifolia* was distributed in Naltar and Hunza valley of Gilgit-Baltistan Pakistan.

A. austriaca, *A. biennis*, *A. pontica*, *A. montana* and *A. tournefortiana* were found in Bagrote, Hunza and Shigar valley of Gilgit-Baltistan Pakistan.

Hunza valley was the dominant area for the diversity of *A. vulgaris*. Ghizer district is dominant area for *A. verlotiorum* and *A. sieversiana*. Population of *A. maritima* was the characteristic feature of flora of Gilgit-Baltistan area.

Based on the collection data of present study, Figure 4.17 reveals the diversity of *Artemisia* in five districts of Gilgit-Baltistan region of Pakistan. Gilgit district have 44 % diversity of *Artemisia* that is highest recorded value in the studied region. 20% diversity was found in Ghizer and Hunza-Nagar district. 10% was found in Skardu district and only 6 % diversity of *Artemisia* was observed in Astore district of Gilgit-Baltistan region of Pakistan.

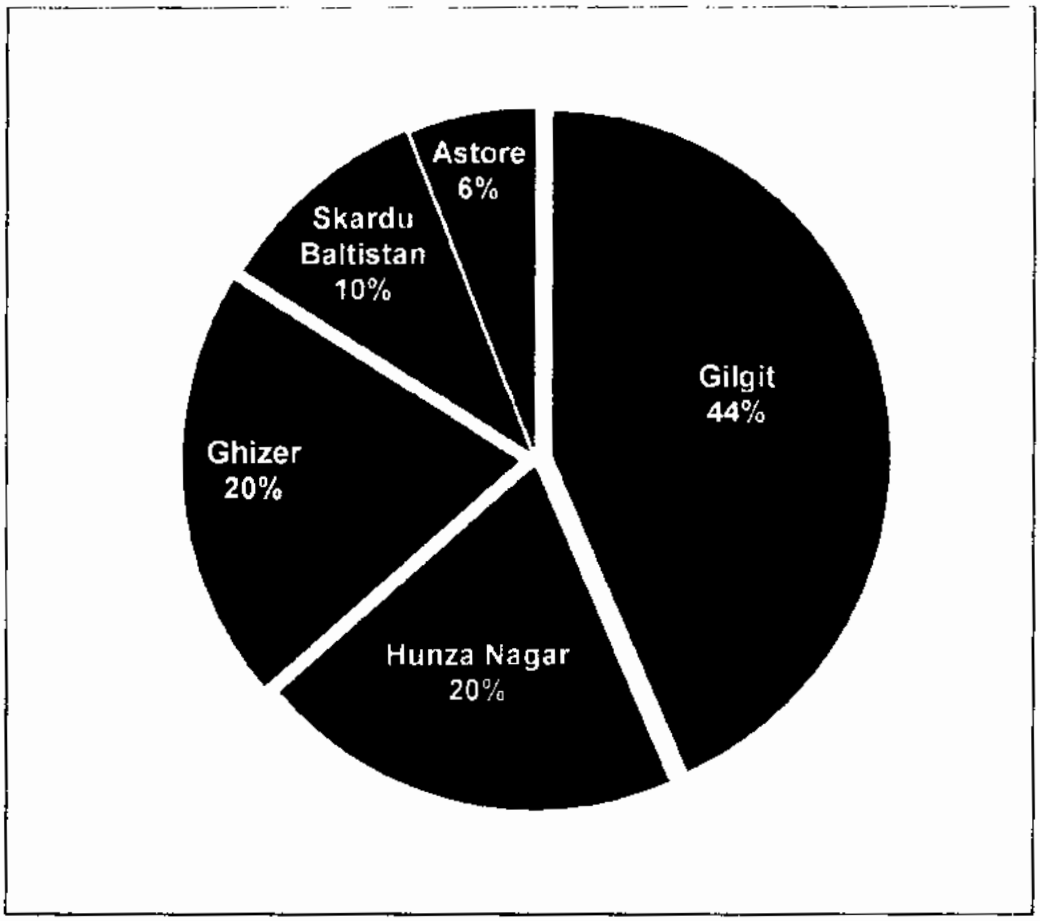

Figure 4.17. *Artemisia* diversity in five districts of Gilgit-Baltistan region of Pakistan.

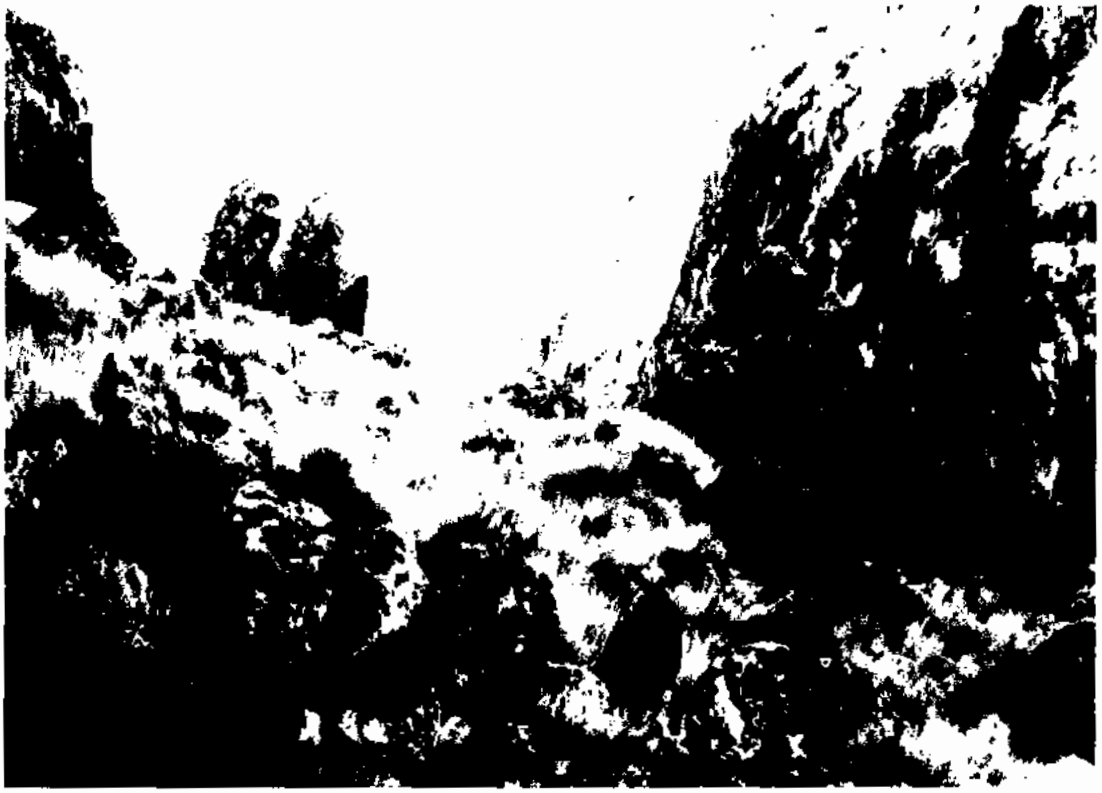

Plate 4.47. *Artemisia herba-alba* **habitat in the mountains of Gilgit-Baltistan region of Pakistan**

Plate 4.48. *Artemisia maritima* habitat in the mountains of Gilgit-Baltistan region of Pakistan

4.6. Molecular Phylogeny

Plates 4.49, 4.50 and 4.51 represents the gel image of ITS, ETS and *psbA-trnH* PCR products of studied *Artemisia* species from Gilgit-Baltistan region of Pakistan (Table 3.1).

In the genus *Artemisia*, the internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA) fragments were ~700 bp in length, while the external transcribed spacer (ETS) of nuclear ribosomal DNA (nrDNA) were ~500 bp long.

The *psbA-trnH* fragments of chloroplast DNA (cpDNA) were ~450 bp in length. After raw sequence processing the final aligned ETS, ITS and *psbA-trnH* sequences are presented respectively, which are used for the phylogenetic reconstruction.

For the ETS, ITS, and *psbA-trnH* sequences, jModelTest predicted HKY+G, 012030+I+G with equal equilibrium base frequencies, and 012010+G as the best model respectively. For CAT64, the best model for the portions representing ETS, ITS, and *psbA-trnH* were HKY+I+G, 012010+G with equal equilibrium base frequencies, and 012010+G respectively.

The numerical data summary from nuclear ribosomal (ITS and ETS) and chloroplast (*psbA-trnH*) DNA sequences for all *Artemisia* samples is presented in Table 4.9. All the tree topologies obtained from independent ML, MP, NJ and Bayesian analyses for ITS, ETS and *psbA-trnH* regions revealed no conflicts between significantly supported (BI-PP>0.95) clades. Merely a little discordance between clades with lower support (BI-PP<0.95) has been noticed, which could be taken as soft incongruences.

The Bayesian, maximum likelihood, maximum parsimony and neighbor joining approaches employed to investigate the pooled nrDNA and cpDNA dataset showed slightly different phylogenetic reconstructions.

The overall monophyly of genus *Artemisia*, including the subgenus *Seriphidium*, was evident and strongly supported in all trees.

In the present study, all clades comprising all subgenera of genus *Artemisia* including *Seriphidium* species were fully supported.

All species of *Seriphidium* were grouped in a monophyletic clade as shown in the MP, ML, NJ and BI trees. The annual *Artemisia* species like *A. annua* formed a sister clade with *Seriphidium*.

The monophyletic groups comprising subgenus *Pacifica*, *Dracunculus* and *Tridentatae* species were placed in separate major clades in all trees.

The results of this study revealed subgenus *Artemisia* and *Absinthium* were appeared as polyphyletic.

This study also observed ten undescribed *Artemisia* taxa in the form of groups (Group I, II and III) in all phylogenetic trees. All clades which comprise undescribed *Artemisia* taxa were also fully supported.

The bootstrap and Bayesian inference values of trees obtained from combined and separate data of three markers with different pylogenetic approches are given below.

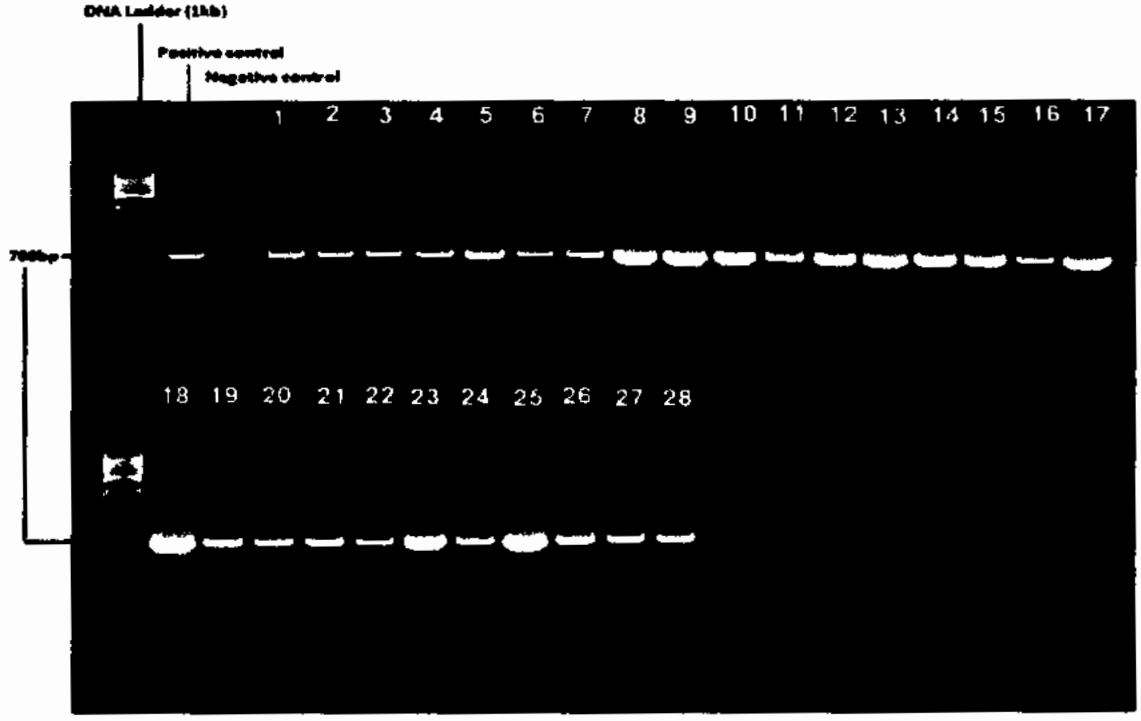

Plate 4.49. Gel image of nuclear ribosomal DNA (nrDNA) ITS PCR products of studied *Artemisia* species from Gilgit-Baltistan region of Pakistan. 1= *A. annua*; 2= *A. arborescens*; 3= *A. argyi*; 4=*A. austriaca*; 5=*A. biennis*; 6= *A. campestris*; 7= *A. chamaemelifolia*; 8= *A. chinensis*; 9= *A.* sp.-AD-H; 10= *A. gmelinii*; 11= *A. herba-alba*; 12= *A. indica*; 13= *A. maritima*; 14= *A. rutifolia*; 15= *A. scoparia*; 16= *A. sieberi*; 17= *A. tournefortiana*; 18= *A. verlotiorum*; 19= *A. vulgaris*; 20= *A.* sp.-A; 21= *A.* sp.-B; 22= *A.* sp.-C; 23= *A.* sp.-D; 24= *A.* sp.-E; 25= *A.* sp.-F; 26= *A.* sp.-G; 27= *A.* sp.-H; 28= *A.* sp.-I

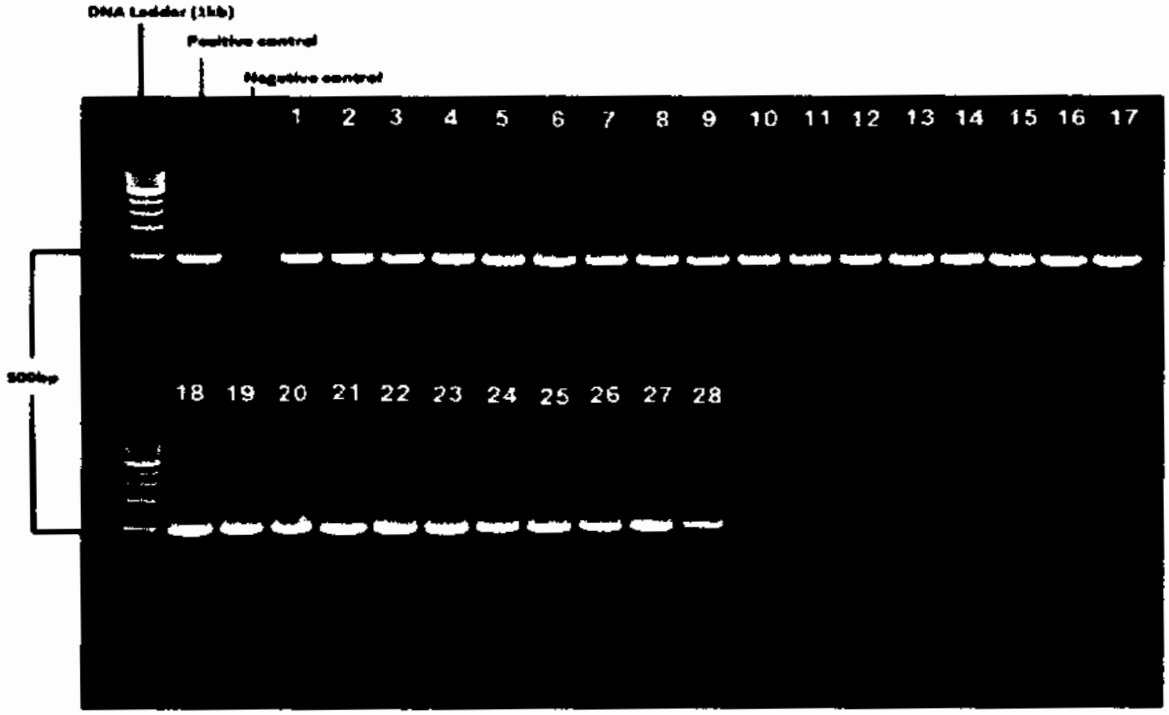

Plate 4.50. Gel image of nuclear ribosomal DNA (nrDNA) ETS PCR products of studied *Artemisia* species from Gilgit-Baltistan region of Pakistan. 1= *A. annua*; 2= *A. arborescens*; 3= *A. argyi*; 4=*A. austriaca*; 5=*A. biennis*; 6= *A. campestris*; 7= *A. chamaemelifolia*; 8= *A. chinensis*; 9= *A.* sp.-AD-H; 10= *A. gmelinii*; 11= *A. herba-alba*; 12= *A. indica*; 13= *A. maritima*; 14= *A. rutifolia*; 15= *A. scoparia*; 16= *A. sieberi*; 17= *A. tournefortiana*; 18= *A. verlotiorum*; 19= *A. vulgaris*; 20= *A.* sp.-A; 21= *A.* sp.-B; 22= *A.* sp.-C; 23= *A.* sp.-D; 24= *A.* sp.-E; 25= *A.* sp.-F; 26= *A.* sp.-G; 27= *A.* sp.-H; 28= *A.* sp.-I

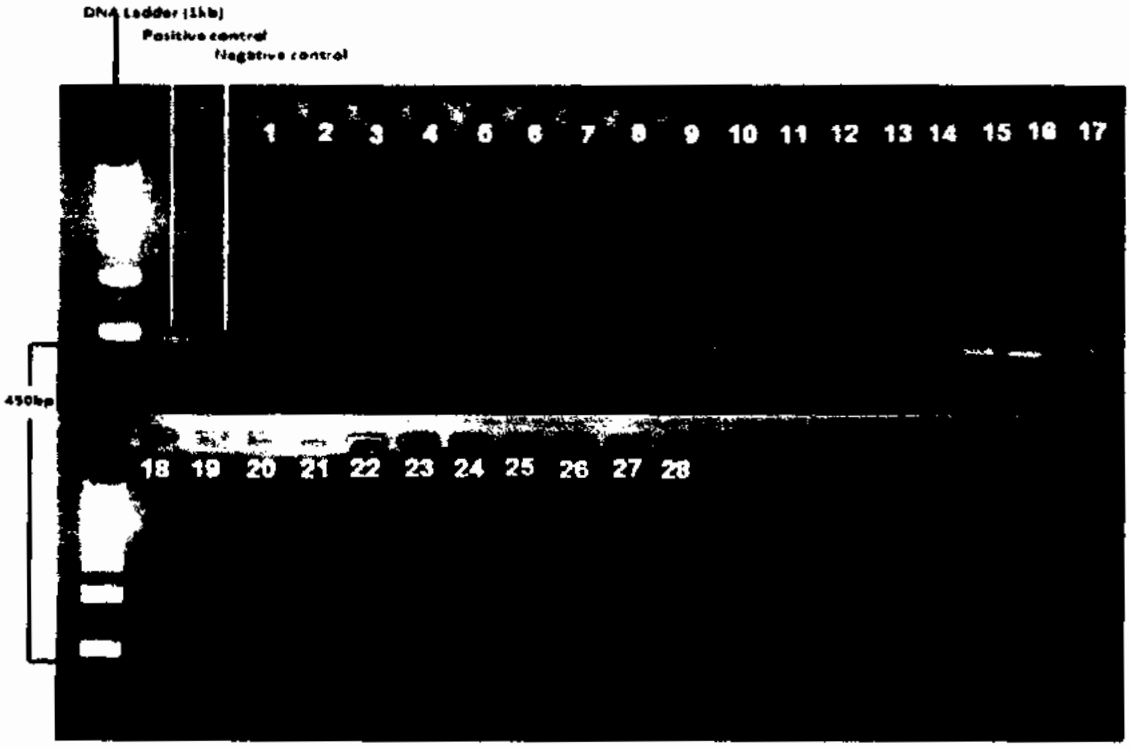

Plate 4.51. Gel image of chloroplast DNA (cpDNA) psbA-trnH PCR products of studied *Artemisia* species from Gilgit-Baltistan region of Pakistan. 1= *A. annua*; 2= *A. arborescens*; 3= *A. argyi*; 4=*A. austriaca*; 5=*A. biennis*; 6= *A. campestris* L; 7= *A. chamaemelifolia*; 8= *A. chinensis*; 9= *A. sp.*-AD-H; 10= *A. gmelinii*; 11= *A. herba-alba*; 12= *A. indica*; 13= *A. maritima*; 14= *A. rutifolia*; 15= *A. scoparia*; 16= *A. sieberi*; 17= *A. tournefortiana*; 18= *A. verlotiorum*; 19= *A. vulgaris*; 20= *A.* sp.-A; 21= *A.* sp.-B; 22= *A.* sp.-C; 23= *A.* sp.-D; 24= *A.* sp.-E; 25= *A.* sp.-F; 26= *A.* sp.-G; 27= *A.* sp.-H; 28= *A.* sp.-I

Table 4.9. Summary statistics from the nrDNA (ITS & ETS) and the cpDNA (*psbA-trnH*) dataset of genus *Artemisia*. The numbers in brackets indicate the results obtained for nrDNA and cpDNA ingroup dataset of *Artemisia*

Genomic region	**ITS**	**ETS**	***psbA-trnH***	**ITS+ETS+*psba-trnH***
No. of samples	78 (75)	79 (76)	65 (62)	79 (76)
No. of sites	657	397	396	1450
No. of informative sites	322(307)	137(129)	56(54)	515(490)

4.6.1. Maximum Likehood Phylogenetic Trees

Figure 4.18, 4.19, 4.20 and 4.21 represents the maximum likelihood phylogenetic trees based on combined and seperate ITS, ETS and *psbA-trnH* sequences of nrDNA and cpDNA of *Artemisia* from Gilgit-Baltistan region of Pakistan. Only the bootstrap obtained from maximum likelihood tree of combined ITS, ETS and *psbA-trnH* markers are discussed here. In the resulting combined ML tree, the overall monophyly of genus *Artemisia*, including the subgenus *Seriphidium* is evident and strongly supported (ML-BS=100 %) (Figure 4.21). Maximum backbone nodes revealed better support (ML-BS>50 %), except few lineages displayed poorly determined nodes.

Subgenus *Artemisia* and subgenus *Absinthium* were appeared as polyphyletic (ML-BS=88 %). Subgenera *Seriphidium* (ML-BS=84 %), *Tridentatae* (ML-BS=97 %), *Pacifica* (ML-BS= 100 %) and *Dracunculus* (ML-BS=96 %) were monophyletic and are indicated by coloured shapes/lines. Taxa with "S" symbolize *Artemisia* from Gilgit-Baltistan region of Pakistan. In this study, ten undescribed taxa of *Artemisia* have been observed. The undescribed taxa were categorized as groups (Group I, II and III) because they share recent common ancestors with known *Artemisia* species. Group I (ML-BS= 83 %). was represented by one undescribed taxon (*A. sp.* -AD-H), 4 undescribed *Artemisia* taxa (*A.* sp. -A, *A.* sp. -B, *A.* sp. -C and *A.* sp. -E) were placed in group II (ML-BS= 98 %). Five undescribed *Artemisia* taxa (*A.* sp. -D, *A.* sp. -F, *A.* sp. -G, *A.* sp. -H and *A.* sp. -I) were presented in the group III (ML-BS= 62 %). In the ML tree based on separate ITS sequences, one undescribed taxon (*A.* sp. -AD-H) with in group I was placed in subgenus *Artemisia* with *Artemisia verlotiorum* as shown in Figure 4.19. While in the ML tree based on separate ETS and *psbA-trnH* sequences, the same undescribed taxon within group I was placed in subgenus *Artemisia* with *Artemisia japonica* as shown in Figure 4.18 and 4.20. In the ML tree of combined ITS, ETS and *psbA-trnH* sequences, the new undescribed taxon from group I was also placed with *Artemisia japonica* as shown in Figure 4.21.

In all ML trees based on combined and separate ITS, ETS and *psbA-trnH* sequences, 4 undescribed taxa within group II were placed in the subgenus *Absinthium* with *Artemisia rutifolia* and designated as *A. rutifolia* complex. Five undescribed taxa within group III were also placed in the subgenus *Absinthium* with *Artemisia sieversiana*.

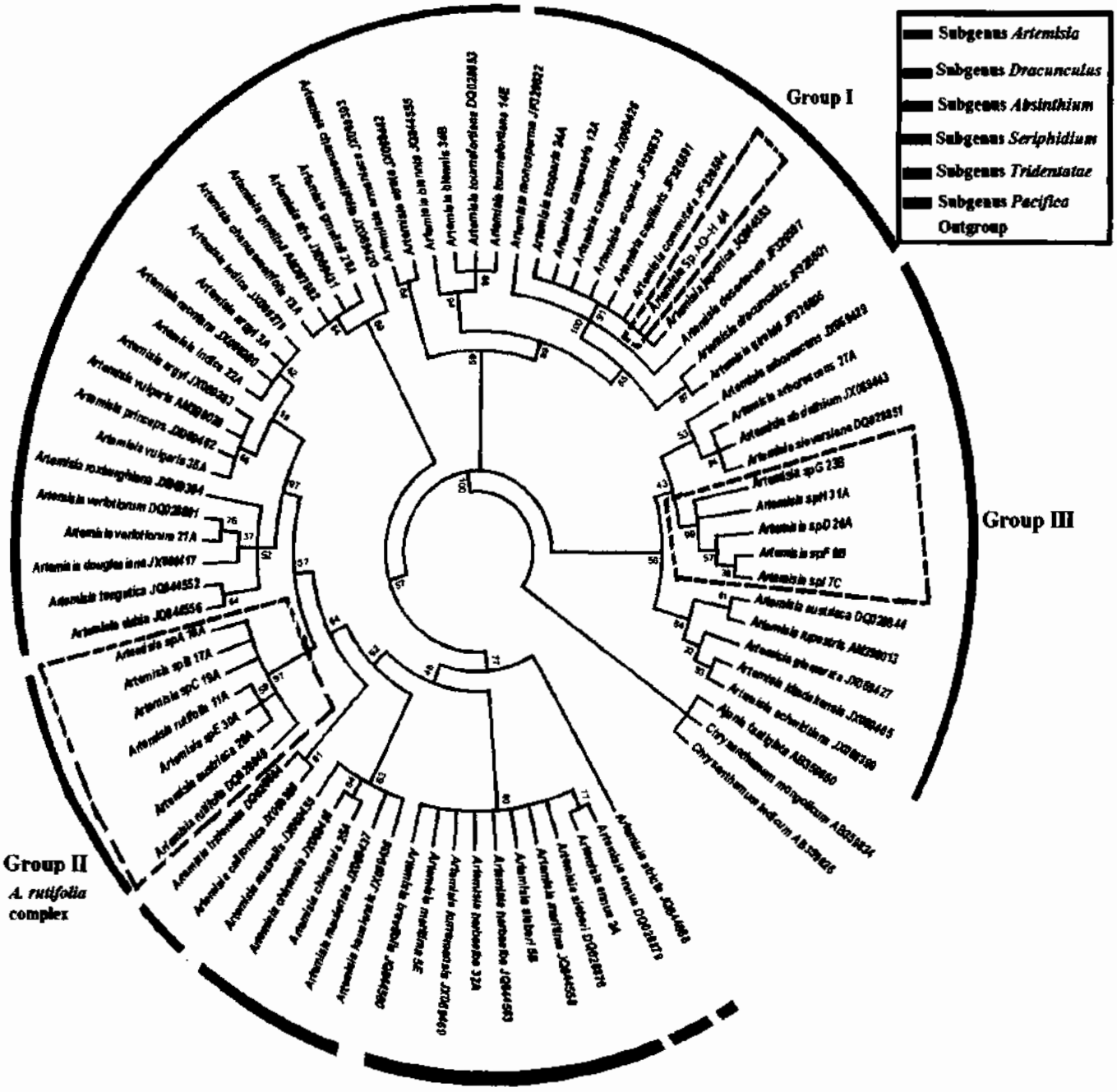

Figure 4.18. Maximum likehood phylogenetic tree based on ETS sequences of nrDNA of *Artemisia* species from Gilgit-Baltistan region of Pakistan. Bootstrap values are indicated along branches. The colored lines drawn specify traditional subgeneric classification of genus *Artemisia*.

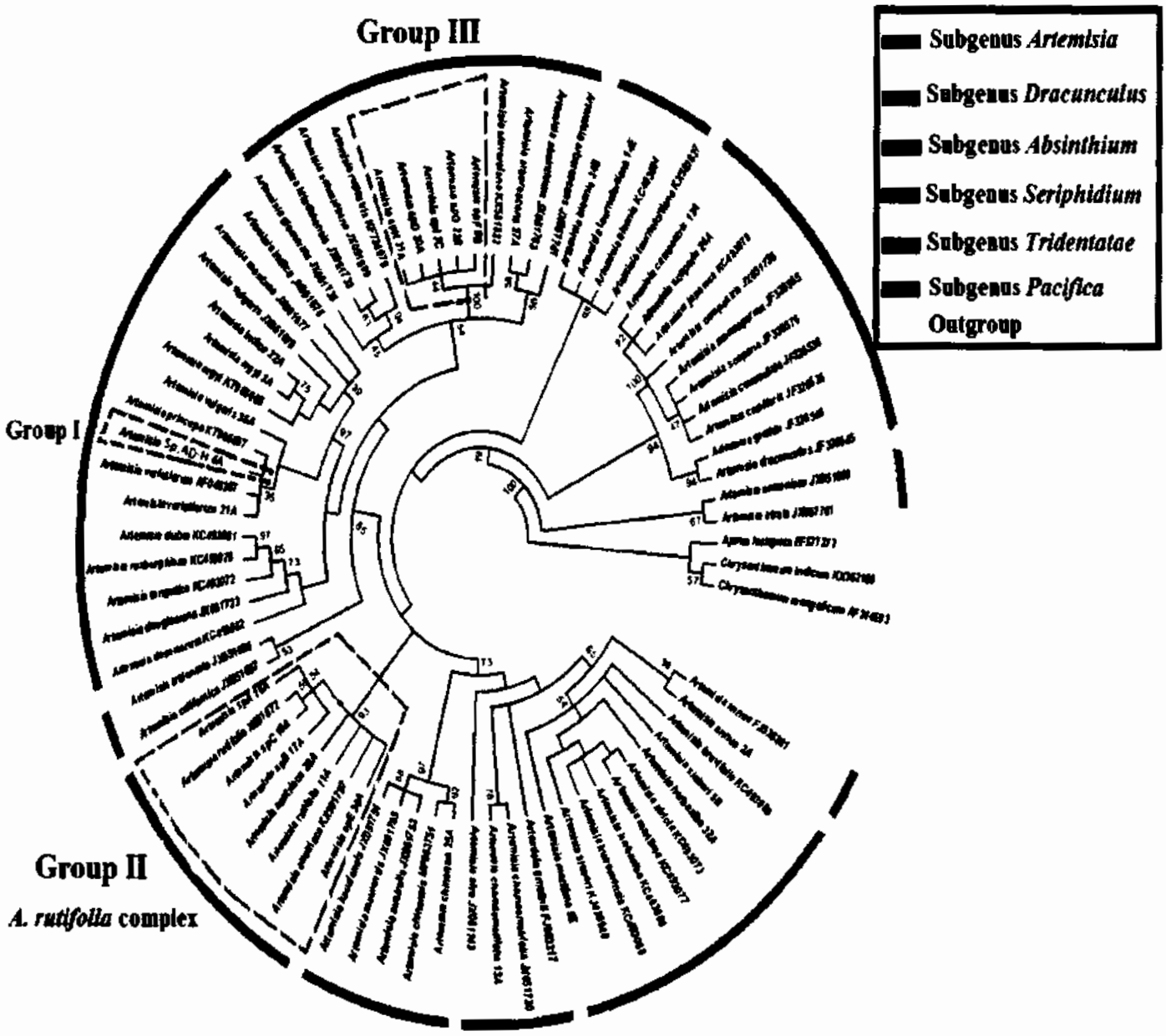

Figure 4.19. Maximum likehood phylogenetic tree based on ITS sequences of nrDNA of *Artemisia* species from Gilgit-Baltistan region of Pakistan. Bootstrap values are indicated along branches. The colored lines drawn indicate the traditional subgeneric classification of genus *Artemisia*.

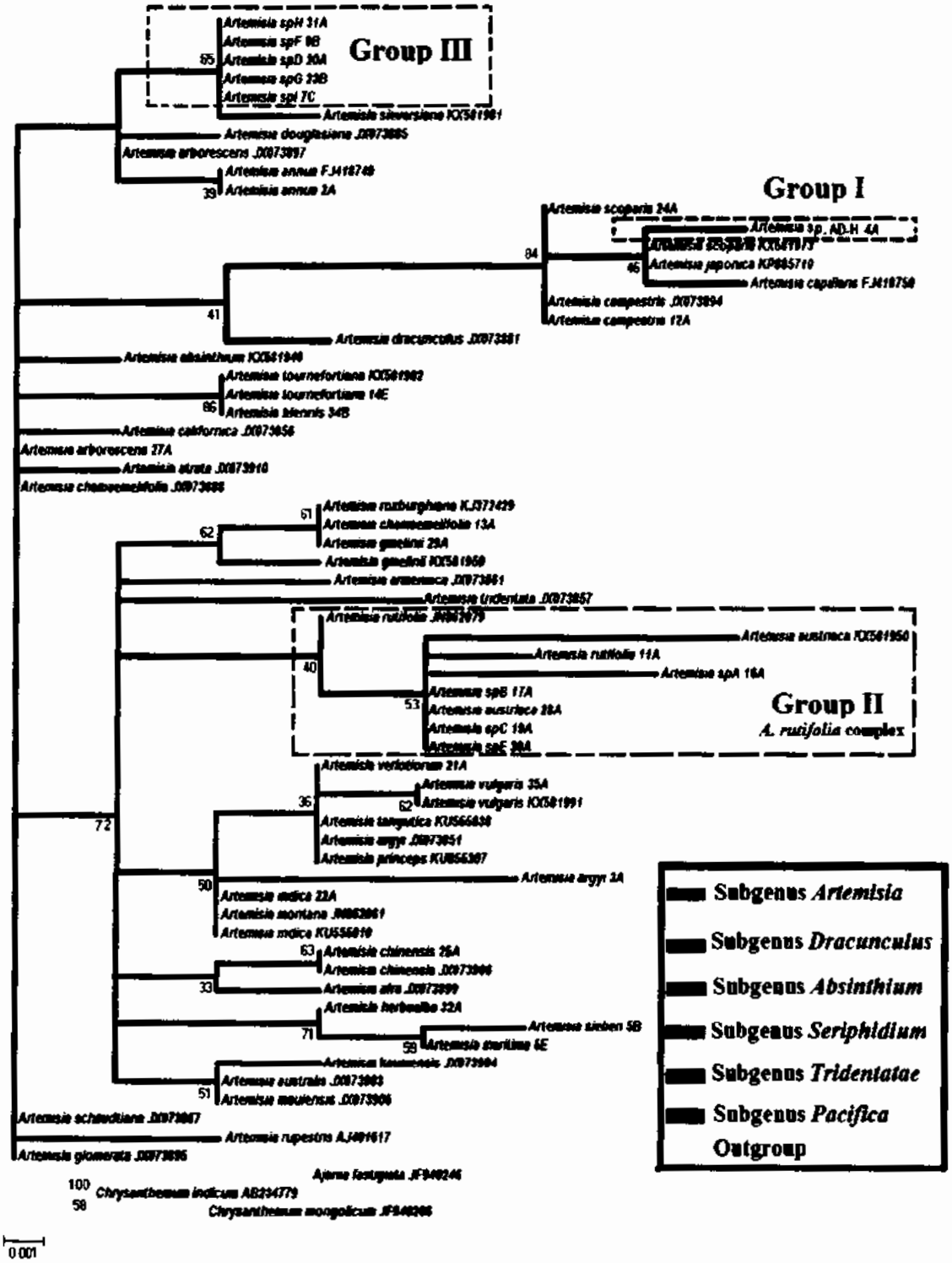

Figure 4.20. Maximum likehood phylogenetic tree based on *psbA-trnH* sequences of cpDNA of *Artemisia* species from Gilgit-Baltistan region of Pakistan. The colored lines drawn show the traditional subgeneric classification of genus *Artemisia*.

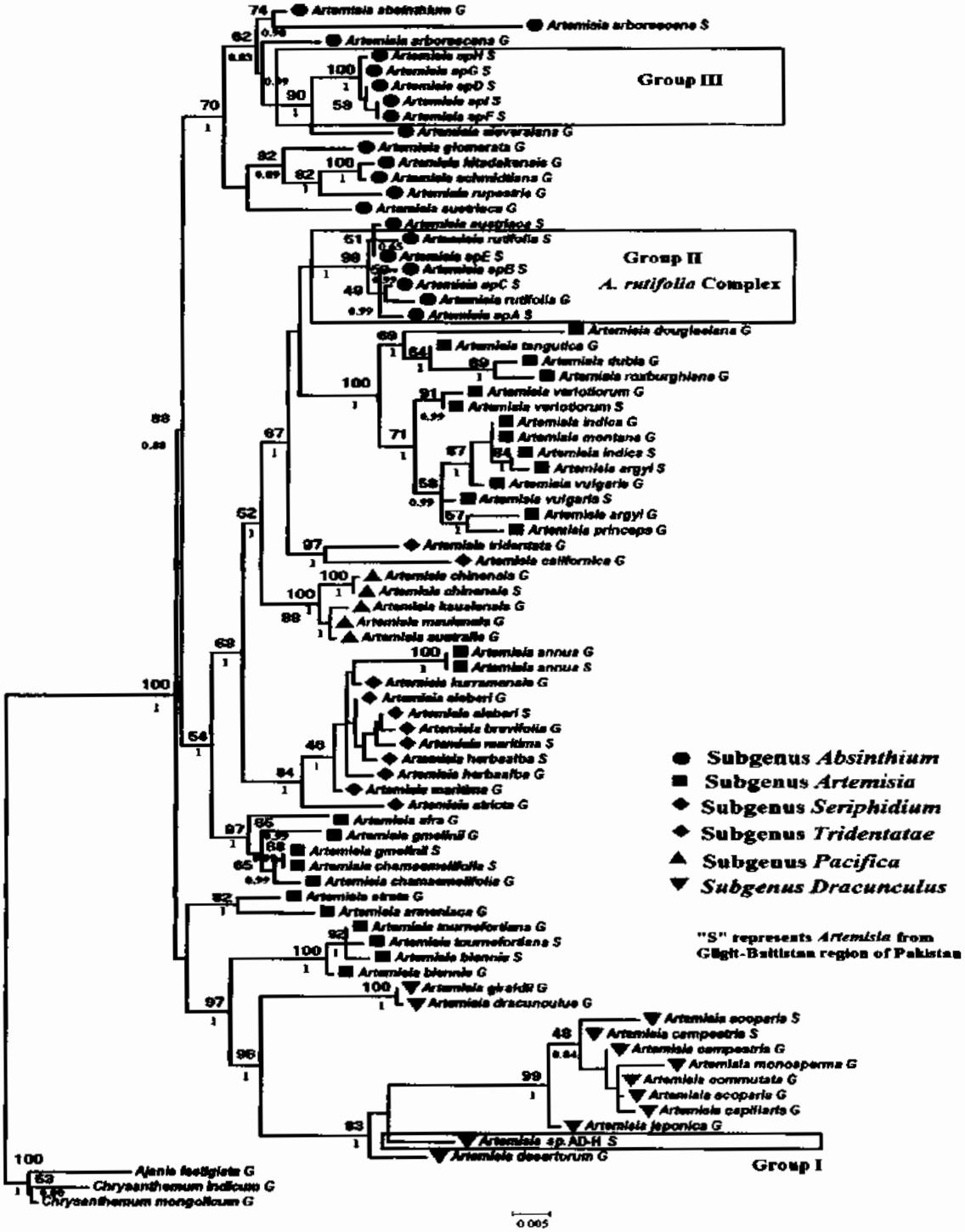

Figure 4.21. Maximum likehood phylogenetic tree based on combined ETS, ITS and *pshA-trnH* sequences of nrDNA and cpDNA of *Artemisia* species from Gilgit-Baltistan region of Pakistan. The values indicated above branches are the Bootstrap values (>50 %) obtained from ML analysis with 1,000 replicates. The values below branches indicate posterior probability (PP) values. The colored shapes specify traditional subgeneric classification of the genus *Artemisia*.

4.6.2. Maximum Parsimony Tree

Figure 4.22, 4.23, 4.24 and 4.25 represents the maximum parsimony phylogenetic trees based on combined and separate ITS, ETS and *psbA-trnH* sequences of nrDNA and cpDNA of *Artemisia* from Gilgit-Baltistan region of Pakistan. Only the bootstrap obtained from maximum parsimony tree of combined ITS, ETS and *psbA-trnH* markers are discussed here. In the resulting MP tree, the overall monophyly of genus *Artemisia*, including the subgenus *Seriphidium* is evident and strongly supported in the combimed MP tree (MP-BS=100 %).

In the resulting combined ITS, ETS and *psbA-trnH* MP tree, maximum backbone nodes revealed better support (MP-BS>50 %), except few lineages displayed poorly determined nodes (Figure 4.25). In the maximum parsimonious analysis, subgenus *Artemisia* and subgenus *Absinthium* were also appeared as polyphyletic (MP-BS= 60 %). Subgenera *Seriphidium* (MP-BS=97 %), *Tridentatae* (MP-BS=88 %), *Pacifica* and *Dracunculus* (MP-BS=80 %) were found to be monophyletic as indicated in Figures by coloured shapes/circles. Taxa with "S" are *Artemisia* from Gilgit-Baltistan region of Pakistan.The maximum parsimony analysis of combined marker genes also showed undescribed taxa within the studied taxa. The undescribed taxa were categorized as groups (Group I, II and III) in all tees because they share common ancestors with known *Artemisia* species. The group I (MP-BS= 100 %) was represented by only one undescribed taxon (*A.* sp. -AD-H). 4 undescribed taxa of *Artemisia* (*A.* sp. -A, *A.* sp. -B, *A.* sp. -C and *A.* sp. -E) were included in group II (MP-BS=76 %). Five undescribed taxa of *Artemisia* (*A.* sp. -D, *A.* sp. -F, *A.* sp. -G, *A.* sp. -H and *A.* sp. -I) were included in group III (MP-BS=65 %). In the MP tree based on separate ETS and *psbA-trnH* sequences, one new undescribed taxon within group I was placed in subgenus *Dracunculus* with *Artemisia japonica* and *Artemisia scoparia* as shown in Figure 4.22 and Figure 4.24, except for the MP tree with separate ITS sequences where this one undescribed taxon within groupI was placed with *Artemisia verlotiorum* as shown in Figure 4.23. In the MP tree based on combined and separate ITS, ETS and *psbA-trnH* sequences, 4 new undescribed taxa of *Artemisia* within group II were placed in the subgenus *Absinthium* with *Artemisia rutifolia* and designated as *A. rutifolia* complex. 5 new undescribed taxa within group III were also placed in the subgenus *Absinthium* with *Artemisia sieversiana*.

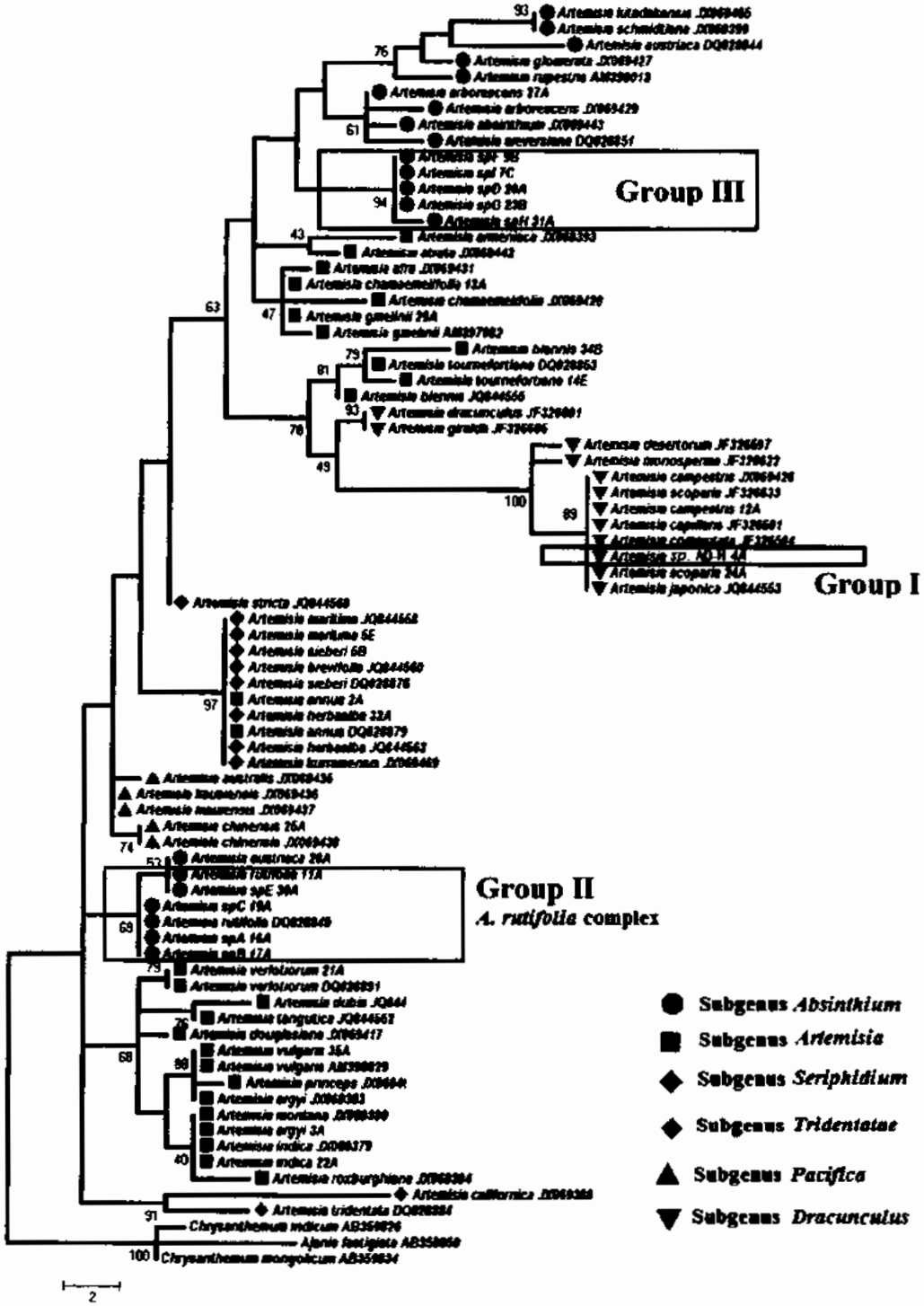

Figure 4.22. Maximum Parsimony phylogenetic tree based on ETS sequences of nrDNA of *Artemisia* species from Gilgit-Baltistan region of Pakistan. Bootstrap values are indicated along branches. The colored shapes drawn specify the traditional subgeneric classification of genus *Artemisia*.

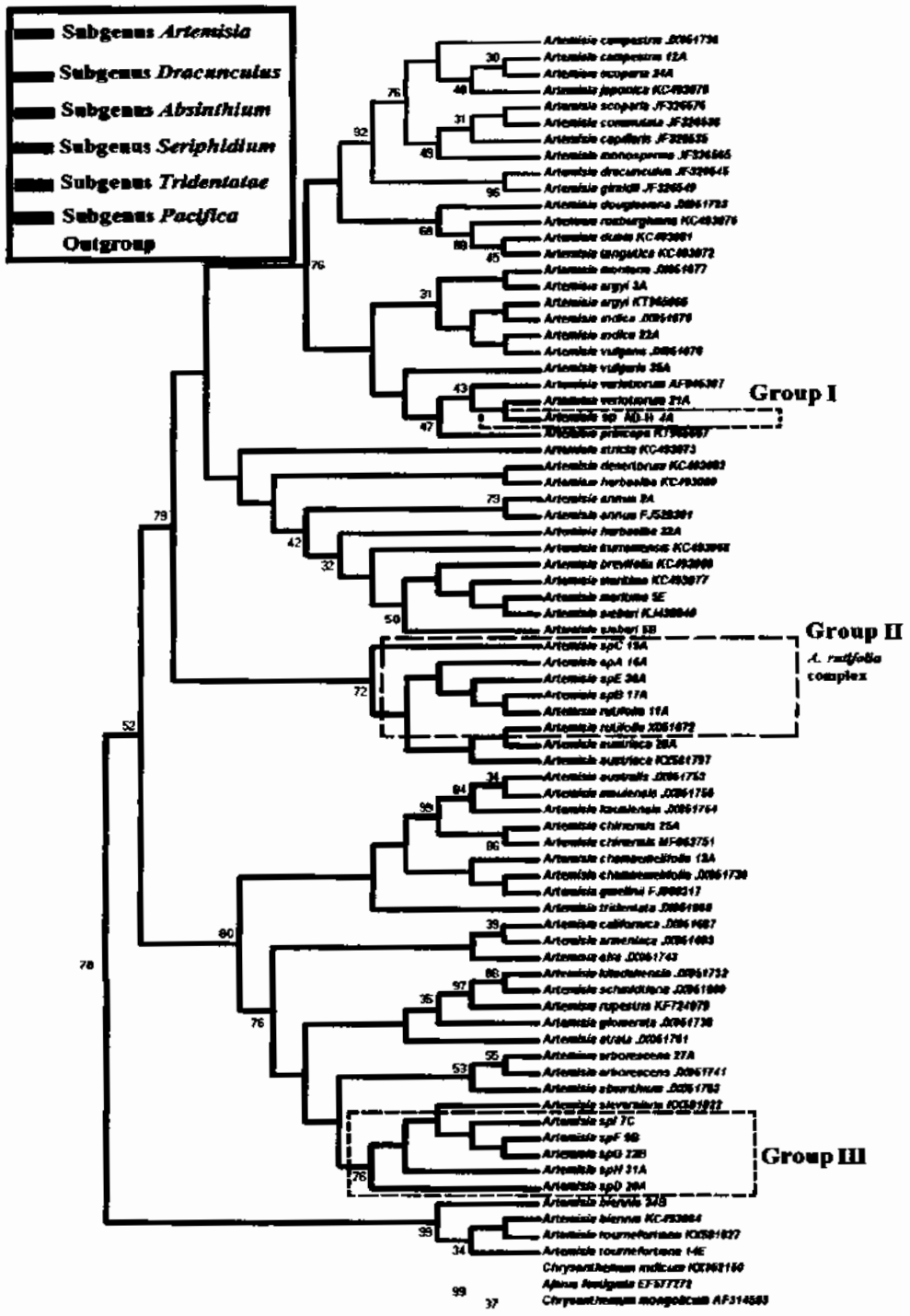

Figure 4.23. Maximum Parsimony phylogenetic tree based on ITS sequences of nrDNA of *Artemisia* species from Gilgit-Baltistan region of Pakistan. Bootstrap values are indicated along branches. The styles of the strokes that draw the branches show the traditional subgeneric classification of genus *Artemisia*.

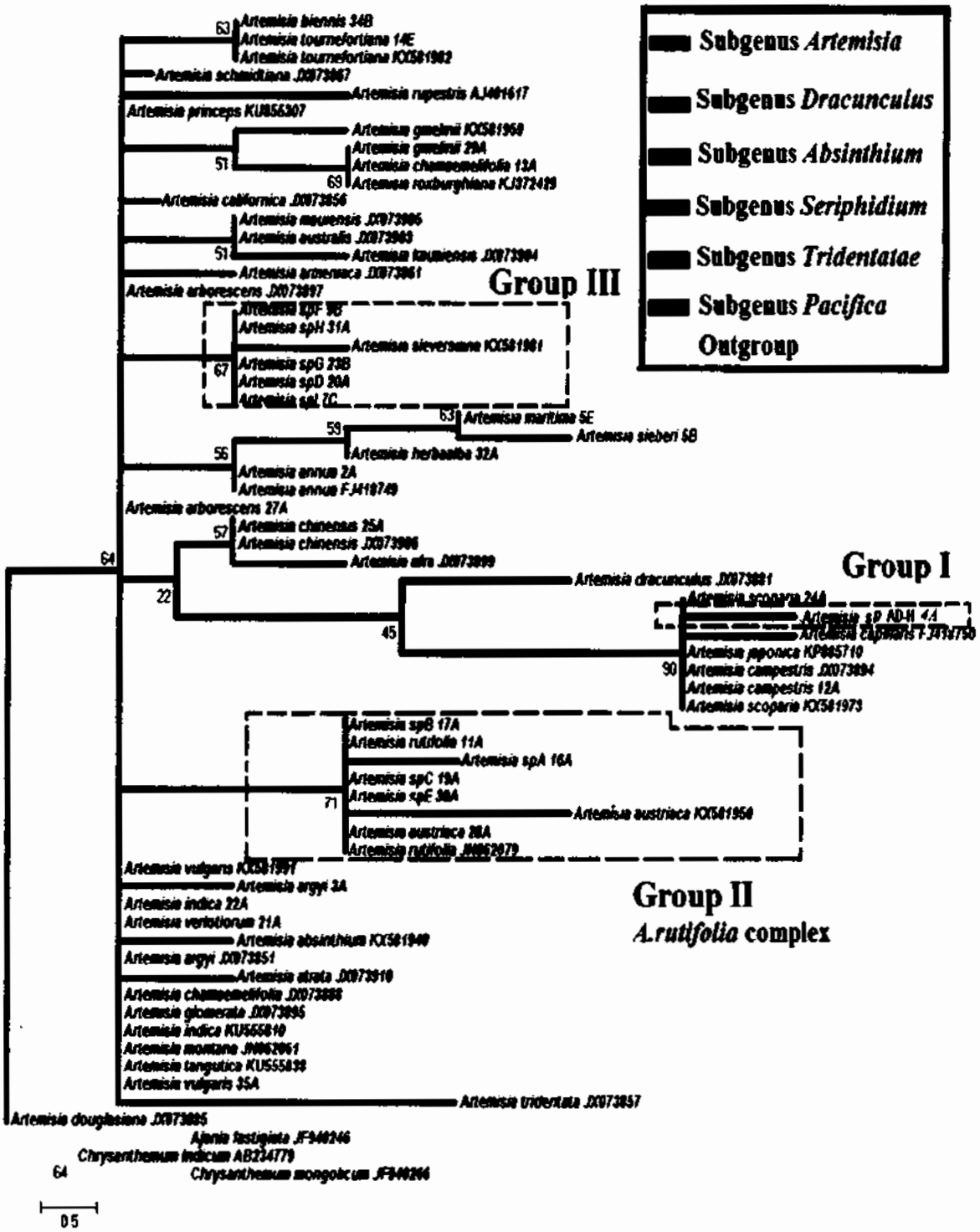

Figure 4.24. Maximum Parsimony phylogenetic tree based on *psbA-trnH* sequences of cpDNA of *Artemisia* species from Gilgit-Baltistan region of Pakistan. Bootstrap values are indicated along branches. The styles of the strokes that draw the branches specify traditional subgeneric classification of genus *Artemisia*.

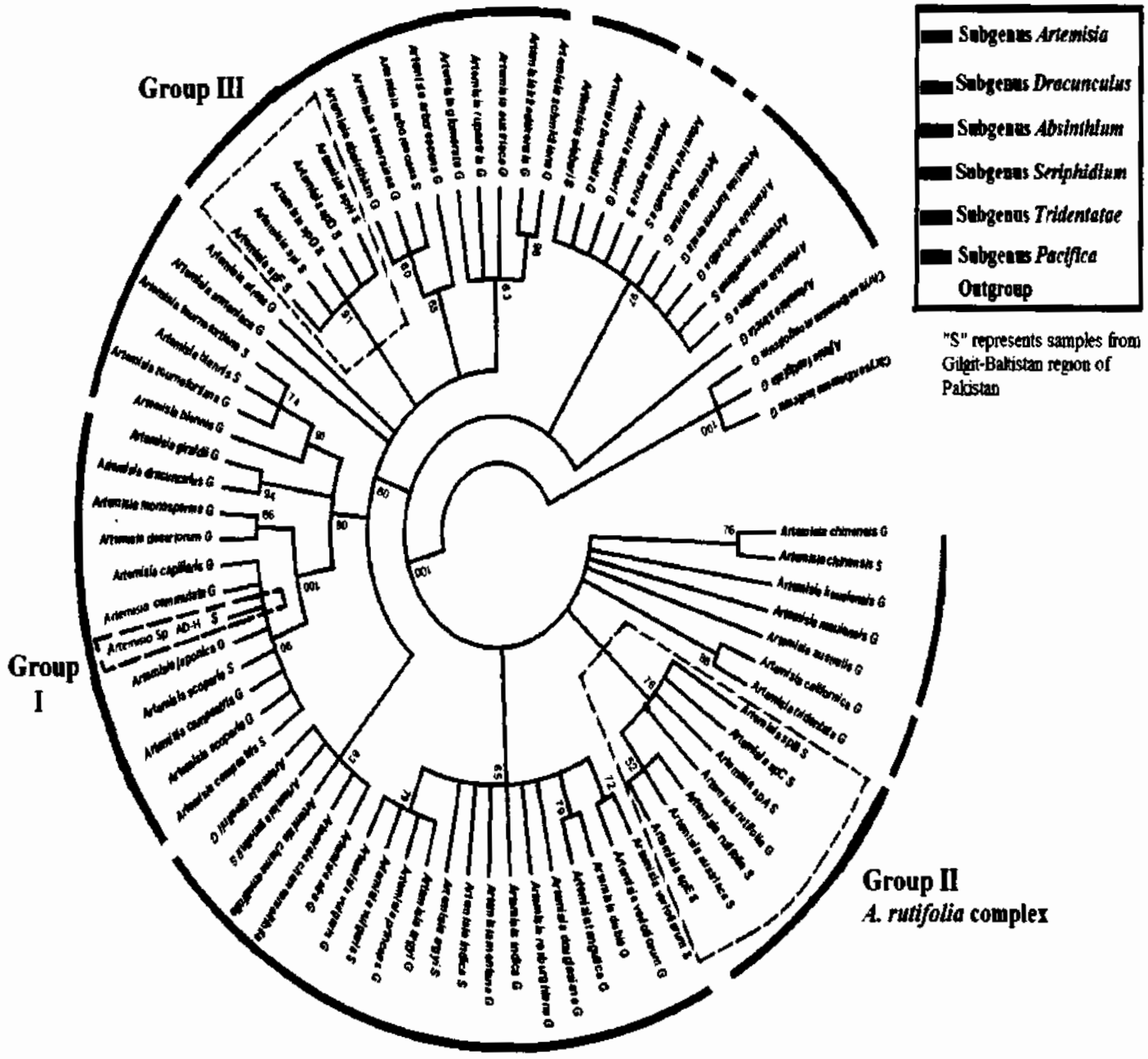

Figure 4.25. Maximum Parsimony phylogenetic tree of combined ITS, ETS and *psbA-trnH* sequences of nrDNA and cpDNA of *Artemisia* species from Gilgit-Baltistan region of Pakistan. Bootstrap values are indicated along branches. The colored lines drawn indicate the traditional subgeneric classification of genus *Artemisia*.

4.6.3. Neighbor Joining Consensus Tree

Figure 4.26 explains neighbour joining consensus tree with boot strap % values based on combined ITS, ETS and *psbA-trnH* sequences of 79 taxa of *Artemisia* from Gilgit-Baltistan region of Pakistan and other parts of the world. The bootstrap obtained from neighbour joining tree of combined ITS, ETS and *psbA-trnH* markers are discussed here. In the resulting NJ tree, subgenus *Artemisia* and subgenus *Absinthium* (NJ-BS= 100 %) also appeared as polyphyletic.

Subgenera *Seriphidium* (NJ-BS= 96 %), *Tridentatae* (NJ-BS=91 %) *Pacifica* (NJ-BS= 59 %) and *Dracunculus* (NJ-BS=70 %) were found to be monophyletic as specified in figures by coloured strokes. Taxa with "S" are *Artemisia* from Gilgit-Baltistan region of Pakistan.

The neighbour joining consensus tree also showed new undescribed taxa. The new undescribed taxa were categorized as groups (Group I, II and III).

In the NJ tree based on combined ITS, ETS and *psbA-trnH* sequences, one undescribed taxon of *Artemisia* (A. sp. -AD-H) with in group I (NJ-BS= 90 %) was located in subgenus *Dracunculus* with *Artemisia japonica*.

Four undescribed taxa (*A.* sp. -A, *A.* sp. -B, *A.* sp. -C and *A.* sp. -E) within group II (NJ-BS= 75 %) were included in the subgenus *Absinthium* with *Artemisia rutifolia* and designaed as *A. rutifolia* complex. Five undescribed taxa (*A.* sp. -D, *A.* sp. -F, *A.* sp. -G, *A.* sp. -H and *A.* sp. -I) within group III (NJ-BS= 100 %) were also placed in the subgenus *Absinthium* with *Artemisia sieversiana* as shown in Figure 4.26.

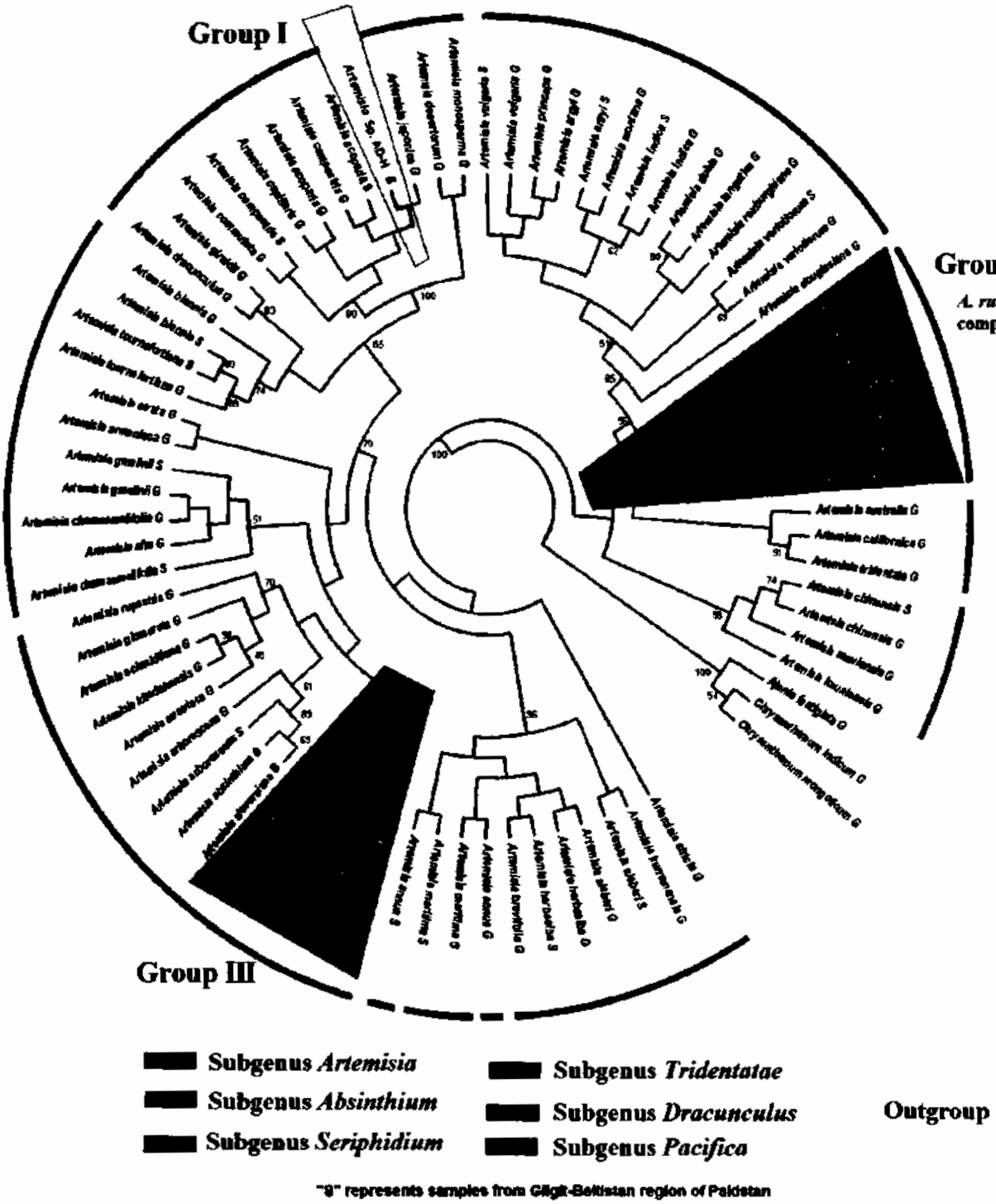

Figure 4.26. Neighbour-Joining consensus tree based on combined ETS, ITS and *psbA-trnH* sequences of nrDNA and cpDNA of *Artemisia* from Gilgit-Baltistan region of Pakistan. Bootstrap values are indicated along branches. The colored lines drawn indicate the traditional subgeneric classification of genus *Artemisia*.

4.6.4. Bayesian Tree

Figure 4.27 explains Bayesian tree of 79 taxa of *Artemisia* based on combined ITS, ETS and *psbA-trnH* sequences from Gilgit-Baltistan region of Pakistan and other parts of the world (Tkach *et al.*, 2007). In the Bayesian analysis, the subgenera *Artemisia* and *Absinthium* were also appeared as polyphyletic (PP= 1). Subgenera, *Seriphidium* (PP= 0.98), *Tridentatae* (PP= 1), *Pacifica* (PP= 1) and *Dracunculus* (PP= 1) were found to be monophyletic as indicated in Figure 4.27 by coloured strokes. Taxa with "S" signify *Artemisia* from Gilgit-Baltistan region of Pakistan.

In the Bayesian analysis based on posterior probabilities (PP), three groups of new undescribed taxa have been observed. One new undescribed taxon (PP= 0.92) within group I was found in the subgenus *Dracunculus* with *Artemisia desertorum*.Four new undescribed taxa of *Artemisia* (PP= 1) within group II were placed in the subgenus *Absinthium* with *Artemisia rutifolia* and designated as *A. rutifolia* complex. Five new undescribed taxa of *Artemisia* (PP= 1) within group III were also placed in the subgenus *Absinthium* with *Artemisia sieversiana* clade as shown in Figure 4.27.

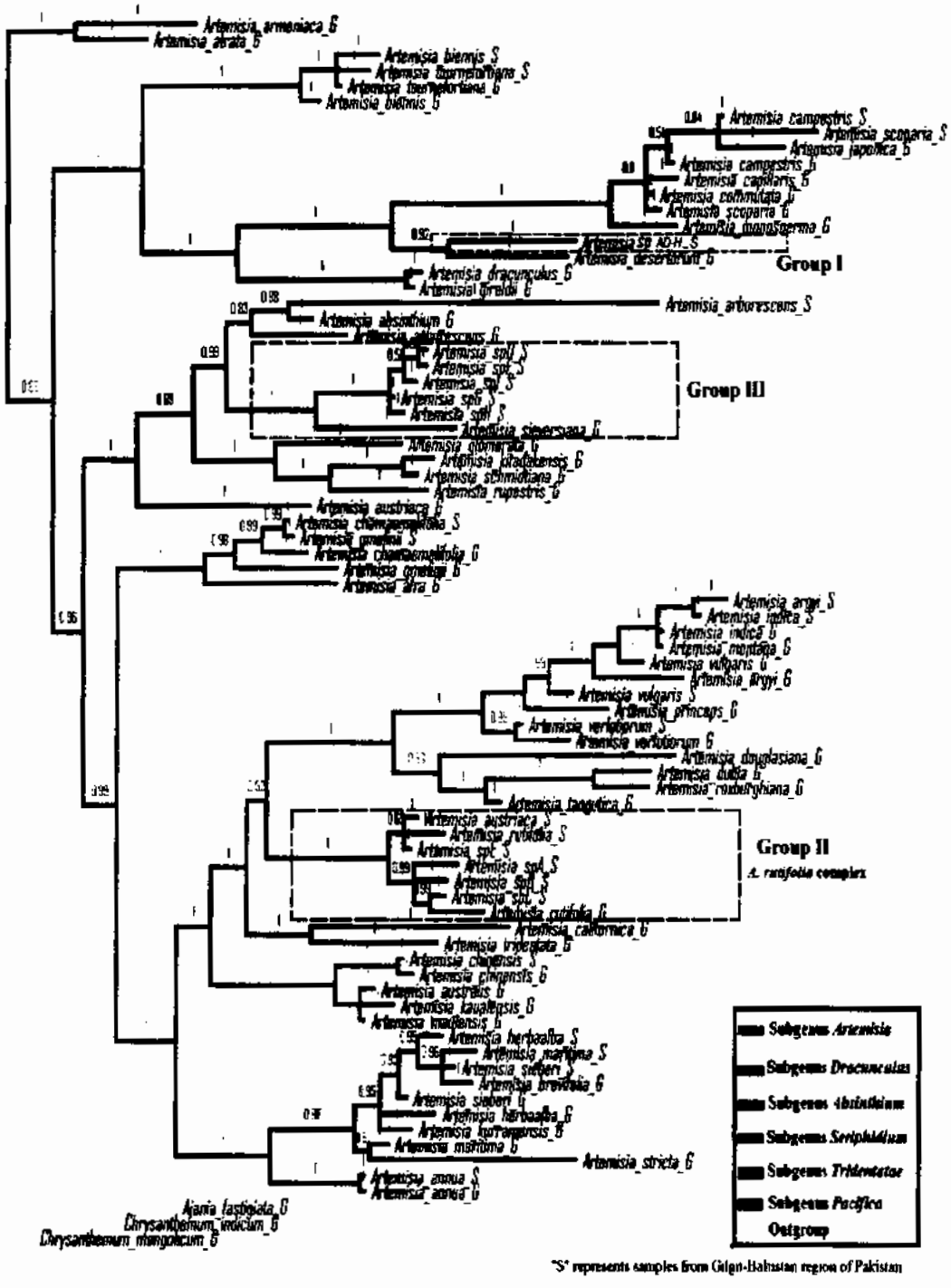

Figure 4.27. Consensus tree (50% majority-rule) from Bayesian inference of the ITS, ETS and *psbA-trnH* sequences of nrDNA and cpDNA dataset of *Artemisia* from Gilgit-Baltistan region of Pakistan. Posterior probability values (PP) are indicated along branches. The styles of the strokes that draw the branches indicate the traditional subgeneric classification of genus *Artemisia*.

CHAPTER 5

5. DISCUSSION

5.1. Ethno botany of *Artemisia*

Several researchers investigated the ethno botanical aspects of important *Artemisia* plants from different regions of Pakistan (Hayat *et al.*, 2009; Ashraf *et al.*, 2010; Fahad *et al.*, 2012; Nadeem *et al.*, 2013). *Artemisia* plants are not merely cultivated for food purposes, but are also employed as folk medicine means. Due to the presence of valuable phytochemical constituents, crude extracts of *Artemisia* showed antioxidant and antibacterial activity, and were found quite effective against certain types of ailments.

Qureshi *et al.*, (2006) acknowledged the vital importance of medicinal plants in different areas of Gilgit-Baltistan. In their final remarks they mentioned a dire necessity for development of conservation approaches in the area that fulfil local needs and economic development opportunities. On the other hands, utilization of plants as medicine poses devastating threats on the plant population and its growth in nature Ghimire *et al.*, 2008).

This study explored 15 rare *Artemisia* species from Gilgit-Baltistan region of Pakistan with their potential folk medicinal uses.

The results of this study confirm other investigations conducted in different regions of Pakistan on genus *Artemisia*. In the far flung regions of Pakistan, various *Artemisia* species have been discovered and evaluated by researchers with a focus on ethno botany. Interviews with native people revealed that the use of *Artemisia* species as traditional medicines is a common exercise there (Hayat *et al.*, 2009).

This study reported *A. scoparia* as a purgative and anti-inflammatory agent. Studies of Hayat *et al.*, (2009) also reported *A. scoparia* as a purgative and also employed for the treatment of burns in Attock city of Pakistan. In this study, most of the species of *Artemisia* were utilized against stomach ailments like stomach pain, diarrhoea and stomach worm.

Hayat *et al.*, (2009) also showed that *A. absinthium* is used for stomach related ailments including stomach worm.

In kurram agency of Pakistan, *A. brevifolia* is utilized as anthelmintic and stomach problems are also treated with this plant (Gilani *et al.*, 2003).

Qurashi *et al.*, (2006) revealed both *A. maritima* and *A. absinthium* as potential medicinal plants of Gilgit region of Pakistan.

Studies in Chitral valley of Pakistan, found that *A. maritima* is used to cure fever stomach pain and intestinal worms (Aziz, 1996).

Another study from Chitral region (Ali & Qaiser, 2009) showed that against certain gastrointestinal complications *Artemisia rutifolia* is employed. Moreover, stomach worms and fever is also cured with this plant.

Ahmed *et al.*, (2006) showed that *A. japonica* and *A. santolinifolia* in Chitral are also utilized as folk medicine.

Ethno botanical uses of *A. scoparia* and *A. vulgaris* were discussed by Khan *et al.*, (2003) and Hussain *et al.*, (2006) which are in agreement with the findings of this study.

Different species of *Artemisia* were studied for the presence of artemisinin by Mannan *et al.*, (2008). They screened *A. annua* and suggested that this plant is a better source of artemisinin, which is used as an antimalarial drug. *A. annua* is also used to treat jaundice, fever and employed as a blood purifier (Sardar & Khan, 2009).

Ethnobotanical aspects of *A. brevifolia, A. absinthium, and A. scoparia* were reported by Khan and Khatoon' (2008).

Study of Ashraf *et al.*, (2010) gathered a lot of ethnobotanical aspects of *Artemisia* in Pakistan and corroborated that most of the *Artemisia* species are also employed in other regions of world. They described ethnobotanical facts regarding the traditional medicinal utilization of *Artemisia* in north of Pakistan. In their study, 8 species of *Artemisia* were recorded which have been prevalently used between local residents as traditional therapeutics. The explored species were *A. absinthium, A. brevifolia, A. dubia, A. moorcroftiana, A. japonica, A. maritima, A. vulgaris* and *A. roxburghiana.*

Fahad and Bano, (2010) studied some threatened plants which were gathered from two different heights in Gilgit-Baltistan. They recognized that *A. laciniata* is employed for gall bladder, jaundice, and high fever. For skin infections, leaf paste of *A. maritima* is utilized.

One study found 26 plant species utilized as traditional medicine in Astore valley of GilgitBaltistan Pakistan (Noor *et al.*, 2014).

However, this study disclosed some new aspects of *Artemisia* based therapies. For example, *A. scoparia* and *A. campestris* both are used for the treatment of jaundice. *A. absinthium* and *A. annua* is used against piles and used as flavor enhancer in local foods and also used as an antiaging remedy. *A. chamaemelifolia* is used as an anthlmintic in childern, *A. rutifolia* sub sp. is used to ease labour pain during pregnancy, *A. indica* is used for relieving kidney pain and skin related diseases, *A. austriaca* is employed for pneumonia and chest pain and *A. verlotiorum* is used to treat stomach related ailments and for dental concerns.

5.2. Morphology of *Artemisia*

Investigations on the diverse genus *Artemisia* suggests that the morphological attributes of *Artemisia* are quite complex to study due to the close relatedness with in species causing difficulties in their differentiation (Hayat *et al.*, 2009b). For example, species of the subgenus *Seriphidium* makes it difficult to differentiate due to similarities in their morphology. It can't be denied that one species holds different appearances in its morphology and these differences makes it difficult in the identification. This condition is also evident in *A. vulgaris* complex as delineated by studies of Stewart, (1983).

The results of morphological study of different *Artemisia* species are mostly same with the previous studies conducted by Hayat, (2011). In the present work, it was observed that the *Artemisia* species holds tendency to gain different morphological forms which causes difficulties in their identification. For example, *A. Argyi, A. dubia* and *A. indica* almost shares same morphological features with little difference.

In this study a quite noticeable difference in plant height was observed between different species of *Artemisia*. The plant height noticed was ranged between 20 and 250 cm. The minimum observed plant height was 10-30 cm in *A. herba-alba, A. chinensis, A. capillaris, A. rutifolia, A. austriaca* and *A. maritima*. Some species (*A. biennis, A. montana, A. verlotiorum, A. tournifortiana* and *A. scoparia*) showed maximum height of 200-250 cm. This study observed ridges on the stem of *Artemisia* species. Also a worth noticeable variation in the leaves of *Artemisia* species was documented.

Investigation of the foliar morphology revealed that the leaves of *Artemisia* species were present as lower (Basal), middle and upper (Aerial). Two forms of leaves like

pinnatifid to pinnatisect were noticed in the lower and middle leaves of studied *Artemisia* species. In most of the studied species, the aerial leaves are mostly linear and long as well as trifid. This was observed in *A. vulgaris*. Leaf petiole length of different *Artemisia* species was varied between sessile to 10 cm. The maximum leaf petiole length observed in *A. annua, A. biennis, A. campestris, A. indica, A. scoparia, A. capillaris, A. tournrfourtiana, A. pontica* and *A. chamaemelifolia* was 10 cm because of having highest capitular diameter.

Numbers of ray florets were 5 to 20 in *A. biennis, A. arborescens, A. chinensis* and *A. pontica*. The ray florets corolla length was found to be around about 2 mm. The absence of ray florets in all the species of sub section *Seriphidium* was noticed. The absence or having lower number of ray florets is prominent feature of species of section *Seriphidium* while having maximum disc florets number is worth feature of section *Absinthium*. This study revealed the length of corolla in disc florets to about 1-2.5mm. The cypselas were small in size. The presence of terminal and lateral scar were observed in the cypselas of different species of *Artemisia*. The size of cypselas in majority of *Artemisia* species was 1-1.5 sq. mm. The colour of cypselas observed in most species was light to dark brown.

In the resulting strict consensus cladogram obtained from the morphological attributes of different *Artemisia* species using maximum parsimony analysis showed that the subgenus *Artemisia* and subgenus *Absinthium* both appeared to be polyphyletic and having the origin at more than one points. This morphological cladistics study showed all other subgenera like *Dracunculus* and *Seriphidium* to be monophyletic.

The cladistic study based on morphological attributes are same and in accordance with the previous morphological studies of Hayat, (2011) and the molecular studies of Ling (1991), Korenkeven *et al.*, (1999), Torell *et al.*, (1999), D'Andrea *et al.*, (2003), Pellicer *et al.*, (2010) and Garcia *et al.*, (2011) on sectional divisions.

Nevertheless, this study showed some disagreement with the classification based on classical sectional method because of the appearance of *Artemisia* species with other subgenera. Also the morphological investigation of this study rejects the separation of Seriphidium and authenticates its recombination with genus *Artemisia*.

That's why the results based on morphological investigation of different *Artemisia* species is not in agreement with the studies of by Bremer and Humphries (1993) and Bremer (1994) who separated *Seriphidium* from genus *Artemisia.* In this work, *Seriphidum* was merged within the clade of subgenus *Artemisia* that's why it is reasonable to say that *Seriphidium* is a subgenus of *Artemisia.*

5.3. Foliar Epidermal Anatomy of *Artemisia*

Artemisia species leaves have been analyzed with both light and scanning electron microscopy. The trichomes attributes were considered generally as a fruitful tool for determining the taxonomic affiliations within the genus *Artemisia* but previous studies of Hayat *et al.*, (2010) have demonstrated that the use of SEM for leaf epidermal surface may support taxonomic studies strongly for this genus. Because, SEM discloses some structures clearer than other microscopic techniques by showing distinct aspects of some attributes.

Studies of Bano *et al.*, (2015) revealed Polygonal shaped epidermal cells with straight pattern walls in *A. persica.* Wang *et al.*, (2016) recently studied drought adaptive characteristics of leaf epidermal cells of different *Artemisia* species. Recently, Ivashchenko and Ivanenko (2017) studied the foliar epidermal cells of *A. abrotanum* and found that this species have thick walled epidermal cells.

In the present study, epidermal cell analysis categorized *Artemisia* species in to three groups one with irregular shape (wavy margined), second with elongated shape (smooth margined) and third with polygonal shape (smooth margins). Hayat *et al.*, (2010) and Rabie *et al.*, (2006) disclosed the epidermal cells variation in different *Artemisia* species. Results of this study also agreed with their findings regarding the shapes and arrangements of epidermal cells. But the quantitative measurements of this data are slightly different from their studies. It could be due to the environmental changes faced by different *Artemisia* species. For example, the detail of variation in epidermal cells, of both abaxial and adaxial surfaces, also explained that most of the *Artemisia* species (*A. herba-alba* and *A. maritima*) have elongated smooth walled cells.

Other species of *Artemisia* including *A. argyi, A. pontica* and *A. montana* have polygonal smooth walled cells, Few species like, *A.* sp. AD-H, *A. herba-alba,* and *A. maritima* have elongated cells with smooth margins. while the rest of *Artemisia* spp. *A.*

annua, *A. chamaemelifolia*, *A. tournefortiana*, *A. indica*, *A. gmelinii*, *A. verlotiorum*, *A. vulgaris*, *A. chinensis*, *A. rutifolia*, *A. dubia*, *A. austriaca*, *A. campestris* and *A. scoparia* showed irregular shape.

Most of the new species investigated in this study including *A. chamaemelifolia*, *A. montana*, *A. pontica* and *A. austriaca* were not focused by other researchers since many years for their foliar anatomical studies.

In their study, Hayat *et al.*, (2010), unveiled a lot about the foliar epidermal anatomy of different *Artemisia* species and tried to resolve the sub generic classification of genus *Artemisia*.

In one study the anatomical changes and frequencies of stomata in leaves of few *Artemisia* species were disclosed by Nautiyal and Purohit (1980), on the other hand, the utilization of foliar anatomical characteristicsfor taxonomic purpose in *Artemisia* species were revealed by Rabie *et al.*, (2006), Noorbakhsh *et al.*, (2008) and Saedi *et al.*, (2009).

This study found five different stomata types namely, anisocytic, diacytic, anomocytic, anomotetracytic, and paracytic. These finds are in complete agreement with the previous findings of Hayat *et al.*, (2010), Rabie *et al.*, (2006), Noorbakhsh *et al.*, (2008) and Saedi *et al.*, (2009).

Investigation of stomatal characteristics suggests that such foliar epidermal anatomical features can be served as taxonomic tools to remove the conflicts at different taxonomic levels within the genus *Artemisia*. For an instance, *A. chinensis and A. gmelinii* can be easily distinguished from the rest of species by their Anomotetracytic type of stomata. Similarly, Anisocytic stomata are prominent feature of *A. vulgaris* and *A. verlotiorum*. Diacytic types of stomata are only present in *A. chamaemelifolia*. Paracytic type of stomata are only associated with *A.* sp. -AD-H and *A. campestris* respectively.

5.4. Foliar Trichomes of *Artemisia*

The micromorphological characteristics of foliar trichomes have played an important role in plant taxonomy, especially of particular groups at generic and specific levels; more and more studies in this field have attracted the attention of plant morphologists and systematists to resolve the taxonomic conflicts (Fang and Fan, 1993).

In order to delineate the taxonomic relationship with in the genus *Artemisia*, the features of foliar trichomes are very crucial (Hall and Clements, 1923) and multiple types of trichomes including both glandular and non-glandular have been documented in different *Artemisia* species (Ferreira and Janick, 1995). In this study, most of the investigated *Artemisia* species possess capitate type trichomes. Previous studies by Kelsey, (1984), Ferreira and Janick, (1995), Ascensao and Pais (1987) Lodari *et al.*, (1989) and Hayat *et al.*, (2009) also confirmed that the capitate type of glandular trichomes is common in the genus *Artemisia*.

A worth noticeable variations were recorded for the capitate trichomes of different *Artemisia* species investigated. For example, the capitate type of trichomes in *A. argyi* (Plate 4.40-B), *A. dubia* (Plate 4.42-C), *A. tournefortiana* (Plate 4.40-D), *A. verlotiorum* (Plate 4.40-F), *A. vulgaris* (Plate 4.40-N), *A.* sp. -H. (Plate 4.40-I) and *A.* sp. -I (Plate 4.40-M) were present in both upper and lower surface of the leaves.

These types of trichomes in *Artemisia* were also reported in previous studies of Hayat *et al.*, (2009) confirming their ellipsoidal shape with the division of two halves. The capitate trichomes of few species showed ellipsoid shape with two halves and few *species* did not show the two halves division. In few species the shape of trichome was same and division of halves was not clear.

Several studies also previously reported capitate type trichomes, but in this study, trichomes showed some differences present in different species of *Artemisia* in comparison to the studies conducted by Slone and Kelsey (1985) in *A. tridentata* Nutt., Ferreira and Janick (1995) in *A. annua*, Smith and Kreitner (1982) in *A. ludoviciana*, Ascensao & Pais (1987) in *A. compestris* L., Kelsey (1984) in *A. nova* and Lodari *et al.*, (1989) in *A. princeps*.

In addition to the capitate trichomes, this study also perceived some other types of trichomes which may be important from the taxonomic point of view. Among those trichomes, pluricellular trichomes of *A. annua* (Plate 4.40-A, Plate 4.42-E), *A. chamaemelifolia* (Plate 4.40-C, Plate 4.42-A) and *A.* sp. -G, (Plate 4.40-G) were observed.

Peltate trichomes that look like a ball with multicellular structures were important feature of *A. austriaca* (Plate 4.40-H), *A. chinensis* (Plate 4.40-K, Figure 4.42-B), *A. indica* (Plate 4.42-D) and *A. montana* (Plate 4.40-J). *A. gmelinii* (Plate 4.40-L) having a thin neck

trichomes. Conical type trichomes with a circular base tapering towards the apex were present in *A. chinensis* (Plate 4.41-G) and *A.* sp. -*A* (Plate 4.41-D). Stinging hair sharp non-glandular trichome with a sting like apex were present in *A. argyi* (Plate 4.41-A). Aduncate long with curved or hook like apex trichome are unique feature of *A. indica* (Plate 4.41-F), *A. verlotiorum* (Plate 4.41-E) and *A.* sp. -I (Plate 4.41-B). Unicellular calavate which are short and narrow from the base and thicker at the apex were present in *A. tournefortiana* (Plate 4.41-C).

Unicellular filiform with sharper apex trichomes were found in *A. austriaca* (Plate 4.41-H) and *A.* sp. -H (Plate 4.41-I). Similarly, unicellular tector non-glandular trichomes in clusters form in *A. herba-alba* (Plate 4.41-J) were characteristic features.

The majority of non-glandular trichomes presented in this study were not previously been reported in *Artemisia* species except for the aduncate and unicellular tector non-glandular trichomes. These two types of trichomes were also noticed in few *Artemisia* species in studies of Hayat *et al.*, (2009). However, this study found aduncate curely trichomes in *A. indica* and *A. dubia*, whereas studies of Hayat *et al.*, (2009) showed these types of trichomes in *A. roxbhurgiana*. Moreover, this study found unicellular tector tichomes in *A. herba-alba* whereas studies of Hayat *et al.*, (2009) showed these types of trichomes in *A. dubia*. It clearly indicates that the aduncate curly trichomes and unicellular tector trichomes are commonly found in diferent *Artemisia* species.

5.5. Pollen Morphology of *Artemisia*

The family Asteraceae is a eurypalynous (Erdtman, 1952) and the genera of this family possess zonocolporate pollen type (Sachdeva and Malik, 1986). The characteristic of pollen are important in evolution and specific and generic level classification of Asteraceae family (Zafar *et al.*, 2007). An important character of pollen is spine present in the exine that can be utilized as diagnostic characters in the genera of Asteraceae (Pinar and Donmez, 2000). On the other hand, the morphology of pollen of different Asteraceae genera previously investigated showed that the exine feature of pollen is very significant in taxonomy and classification based on phylogeny (Mbagwu and Edeoga, 2006).

The pollen morphological evolutions have the ability to develop more and more degenerative structures (Hayat *et al.*, 2009; 2010). Few characters of pollen like, Globular

pollen shape, arrangement dense spinules, broad spinule base, granular exine sculpture, large pollen size, thick exine and broad colpus width, these all are the plesiomorphic characteristics of *Artemisia* pollen. On the other hand, in apomorphic condition these pollen characteristics have ability to transformed to oblate pollen, lose arrangement of spinules, without prominent spinule base, sinuolate exine sculpture, small pollen size and volume, reduced exine thickness and thin colpus.

Studies authenticated one reason behind this evolution is the patterns of pollination from entomophily to anemophily. While, climate change patterns with high latitude high elevation to low latitude during the relocation from North Temperate Zone and low evaluation moist regions during the glacial epoch are other major cause of this evolution in pollen of *Artemisia* (Wang, 2004; Jiang *et al.*, 2005).

From the LM and SEM annotations, the shape of pollen grain in the investigated *Artemisia* species was homogeneous. This clearly authenticates the monophyletic nature of the genus *Artemisia*. The monophyly of *Artemisia* was also confirmed in the previous studies of Torrell *et al.*, (1999), Watson *et al.*, (2002) and Hayat *et al.*, (2010). Consequently, this investigation also supports the recombination of subgenus *Seriphidium* with *Artemisia* on the basis of pollen shape and arrangement.

The general features of *Artemisia* pollen showed in the present study is in high concordance with studies reported by Jiang (2005) and Hayat *et al.*, (2010) where they found approximate symmetry or globular, 3 lobed spheres in the equatorial view while ellipsoid in the polar side with tricolporate structure in different species of *Artemisia.*

The reults of this study are in accordance with the previous studies of Hayat *et al.*, (2010) authenticating the hypothesis of Jiang *et al.*, (2005) that suggested that the differentiation in species of *Artemisia* based on their pollen micro-morphological characters is very difficult task. This study also agreed with the conclusions and results of Hayat *et al.*, (2010) and Martin *et al.*, (2001 and 2002) that pollen morphology is a diagnostic feature for *Artemisia* and recognized as excellent taxonomic marker.

5.6. Phytogeography of *Artemisia*

There exist many hypotheses about the migration pathways adopted by *Artemisia* in order to colonize new habitats. Primarily, the Asian species from subgenus *Seriphidium* are

considered to be the possible ancestors of *Artemisia* endemic to the North American and its pathway of migration through the Bering Strait have been reviewed (Ling, 1995).

Secondly, many studies (McArthur and Plummer, 1978; McArthur *et al.*, 1981) agreed with this route of migration, which clearly authenticates that the herbaceous members from subgenus *Artemisia* in Northern America can be differentiated during the Pleistocene due to climatic changes, which gives rise to the subgenus *Tridentatae* species and another species endemic to North America. On the basis of phytochemical data, Jeffrey (1995) also agreed the origin of sub genus *Tridentatae* from subgenus *Artemisia*.

The *Artemisia* species which are endemic to South America befall essentially in Argentina (Ariza, 1997), from where, some were migrated to Chile (Zuloaga *et al.*, 2008) and were utilized as medicine traditionally (Roig, 2002).

The global distribution of *Artemisia* was developed by Tkach *et al.*, (2007) and they divided that distribution into 13 floristic sections. In their study, Tkach *et al.*, (2007) corroborated that the assemblage of *Artemisia* species from Pakistan falls into south-west Asian region. Nearly, 40 *Artemisia* species are found in Pakistan (Ghafoor, 2002), 38 species are available in the flora of Pakistan (Sterwart, 1972). Moreover, 42 *Artemisia* species can be found in the Vascular Plants Annotated Catalogue of Pakistan and Kashmir. These *Artemisia* species are distributed in all five districts of Gilgit-Baltistan regions of Pakistan. This study revealed Gilgit district has 44 % diversity of *Artemisia* that is highest recorded value in the studied region. 20% diversity was found in Ghizer and Hunza Nagar district. 10% was found in Skardu district and only 6 % diversity of *Artemisia* was observed in Astore district of Gilgit-Baltistan region of Pakistan.

According to Hayat, (2011) Pakistan's west Himalayan area holds maximum *Artemisia* species diversity. They found 53% species were in west Himalayan region and 30% Tibetan region. Investigations of Ling (1982-1995) and Wang (2004) suggested that the *Artemisia* population in Pakistan exists due to the Quaternary period diversifications and more dispersion to the West Asian regions from North regions of Asia.

5.7. Molecular Phylogeny of *Artemisia*

Since the last two decades, phylogenetic investigations on the basis of nucleotide sequences from the transcript coding or non-coding loci has shown to be a promising tool

in order to assess the genetic diversity within organisms. Nevertheless, Schlotterer *et al.*, (1994) recommends that the revelation of phylogenetic relations of closely related species can be achieved only by utilizing the regions of DNA as markers.

The data presented in the resulting trees demonstrates subgeneric classification of Northeastern (Gilgit-Baltistan) Pakistani *Artemisia* based on ITS, ETS and *psbA-trnH* marker genes. The trees obtained from combined data of three marker genes indicated that all sampled species of genus *Artemisia* form a well-supported monophyly (PP=1; ML-BS=100 %, MP-BS=100 %, NJ-BS 100 %). From this study, some primary conclusions regarding infrageneric limitations and appearance of some undescribed taxa (Groups) can be made on the emerging pattern of the resultant phylogeny.

In all the combined ITS, ETS and *psbA-trnH* phylogeny obtained from different methods, two subgenera of genus *Artemisia* appears to unsettle their monophyly. i.e. Subgenus *Absinthium*, which appeared as polyphyletic forming two major clades. One clade appeared separately (PP=1; ML-BS=70 %, MP-BS=60 %), while the other clade appeared with species of subgenus *Artemisia* (PP=1; ML-BS=98 %, MP-BS=76 %). The subgenus *Absinthium* is different from other subgenera due to the hairy receptacle.

The subgenus *Artemisia* is also not supported as monophyletic and appeared as polyphyletic with its species placed in five major sister clades with five subgenera including *Absinthium*, *Dracunculus*, *Pacifica* and *Seriphidium*. The subgenus *Artemisia* is apparently defined taxonomically on the basis of plesiomorphies (Heterogamous, disciform capitula with and pistillate ray florets and fertile disk florets) and this subgenus needs to be resumscribed.

In previous revelations, the two subgenera like *Absinthium* and *Artemisia* both were pooled in one subgenus called *Artemisia* (Gray, 1984; Watson *et al.*, 2002; Shultz, 2009). Due to the formation of sister clade with subgenus *Artemisia* species, the subgenus *Absinthium* could be merged within subgenus *Artemisia*. That's why; this study favors Gray, (1884) and Watson *et al.*, (2002) for the unification of these two subgenera.

The origin of subgenus *Sriphidium* was unresolved and once treated as segregate genera (Ling 1982; Bremer, 1994; Bremer & Humphries, 1993; Ling, 1995). The ITS, ETS and *psbA-trnH* phylogeny places *Seriphidium* among the annual *Artemisia* species supporting its reunion with in *Artemisia*.

The reunion of *Seriphidium* with in genus *Artemisia* is strongly supported (PP=1; ML-BS=84 %, MP-BS=97 %, NJ-BS= 96 %) in complete agreement with previous studies (Korenkeven and Watson, 1999; Torell *et al.*, 1999; Watson *et al.*, 2002; D'Andrea et al. 2003; Pellicer *et al.*, 2010; Garcia *et al.*, 2011; Hayat, 2011; Riggins & Seigler 2012; Hobbs and Baldwin, 2013; Malik *et al.*, 2017) and is not in agreement with Ling (1982), Bremer (1994), Bremer & Humphries (1993), Ling (1995) and Haghighi *et al.*, (2014). Here, the phylogenetic reconstruction, showed *Seriphidium* species forming a single monophyletic clade. Nevertheless, Malik *et al.*, (2017) showed *Seriphidium* species with two clades suggesting that this subgenus is not monophyletic. They corroborated that one large monophyletic group was corresponded to the formerly recognized sect. *Seriphidium* and the second small clade was phylogenetically distant from the first. The subgenus *Seriphidium* is characterized by discoid homogamous capitula with bisexual disc florets and no ray florets.

Species from the subgenus *Dracunculus* formed a strongly supported single monophyletic clade (PP=1; ML-BS=96 %, MP-BS=80 %, NJ-BS= 70 %). and is sister to the subgenus *Artemisia* species like *A. biennis* and *A. tournefortiana.* Therefore, this study favors Watson *et al.*, (2002) for retaining *Dracunculus* as subgenera of the genus *Artemisia.* This subgenus possesses heterogamous flower heads with pistillate outer florets and sterile inner florets. Two species from subgenus *Tridentatae* also formed a monophyletic clade with strong support (PP=1; ML-BS=97 %, MP-BS=88 %, NJ-BS= 91 %).

The monophyly of sub genus *Tridentatae* is also confirmed in previous studies (Kornkven *et al.*, 1998; Kornkven *et al.*, 1999; Torrell *et al.*, 1999; Valles *et al.*, 2003)

Species from subgenera *Pacifica* also formed a strongly supported single monopyletic clade (PP= 1; ML-BS= 100 %, NJ-BS= 59 %) and confirms its monophyly which is completely in agreement with Hobbs & Baldwin, (2013) and Malik *et al.*, (2017) retaining it as a subgenera. Further studies of this diverse and large genus *Artemisia* (*s.l.*) is crucial for determining phylogenetic relationships with in the genus as whole.

Besides the infrageneric classification of *Artemisia* (*s.l.*), this phylogenetic investigation observed 10 undescribed taxa as three unique groups (Group I, II and III) in the studied taxa collected from the Northeast (Gilgit-Baltistan) region of Pakistan.

One new undescribed taxon of *Artemisia* in group I appeared with boot support (PP=1; ML-BS=83 %, MP-BS=100 %, NJ-BS= 91 %) with in subgenus *Dracunculus*. Four undescribed taxa of *Artemisia* were appeared as group II with strong boot support (PP=1; ML-BS=98 %, MP-BS=76 %, NJ-BS= 75 %) in the second clade of subgenus *Absinthium*. The undescribed taxa of *Artemisia* within group II were placed with *A. rutifolia* lineage. That's why; this clade was named as *A. rutifolia* complex. In the genus *Artemisia*, previous workers have already reported taxonomic complexes. For example, *A. vulgaris* complex: This complex was extensively described by Kaul and Bakshi (1984) and again reported by Sanz *et al.*, (2008). A detailed morphological study of extensive sampling coupled with modern molecular techniques can resolve the taxa delimitation in the *A. rutifolia* complex which may leads to the identification of new species.

In the first clade of subgenus *Absinthium*, five undescribed taxon of *Artemisia* were also placed in group III with moderate boot support (PP=1; ML-BS=62 %, MP-BS=65 %, NJ-BS= 100%). For the differentiation of a species, if the minimum branch lengths before the terminal node in a clade are compared then it is clear that they all are expected new species. This is because the branch lengths are too long in case of group III. Same is the case for the sample observed as undescribed taxon of *Artemisia* in group I.

Koloren *et al.*, (2016) observed two new haplotypes within *Artemisia* samples including both rare and common ones from the Ordu province of Turkey. In their resulting phylogenetic trees, the two haplotypes were placed with *A. argyi*, *A. sylvatica*, and *A. verlotiorum* of subgenus *Artemisia*.

Additionally, This study approve the conclusions made by Koloren *et al.*, (2016) that the grouping of all undescribed taxa of *Artemisia* disjointedly from each other specifies further polyphasic examinations. Those inquiries must include an extensive number of samples in order to characterize some diverse species or subspecies.

5.8. Conclusion

Based on the results of this research, following conclusions are reasonable to make.

1. Since ancient times, the trend of using medicinal herbs traditionally is still popular and continued among the people of Gilgit-Baltistan. The continuance of this awareness quite evidently validates the persistent dependency on the medicinal plants by the local communities. From the ethno botanical perspectives of this study, 15 *Artemisia* species were found with great folk medicinal prominence. It is clear that the *Artemisia* species from Gilgit-Baltistan are very important medicinally and represent a precious natural asset.

2. Cladistic analysis of morphological characters presented that the capitular morphology is not a trustworthy taxonomic marker; rather it is conventionally proven to be a noteworthy feature for infrageneric classification of *Artemisia*. The cladistic revisions based on morphology of *Artemisia* from Gilgit-Baltistan region of Pakistan exposed that *Artemisia* and *Seriphidium* subgenera are monophyletic.

3. The variation in foliar epidermal cells and the pattern of stomata of *Artemisia* species observed are very useful taxonomically. This investigation unveiled 10 types of leaf trichomes, 5 different types of stomata and 3 types of leaf epidermal cell walls in different *Artemisia* species from Gilgit-Baltistan region of Pakistan. These attributes are valued taxonomic signs in the resolution of issues related to taxonomy. These revelations may essentially further enlighten taxonomist's perceptions in the process of species delimitation in *Artemisia* genus.

4. Investigation based on pollen substantiated that the micromorphological characters of *Artemisia* pollen could be a crucial indication for taxonomic revelations with in the genus. This study exposed the pollen grains of *Artemisia* from different regions of Gilgit-Baltistan to be tricolporate (Ellipsoid ball like from equatorial view and round 3-lobed from polar side) with few exceptions and pollen surface is protected by reduced spinules. SEM study corroborated that these spinules are unique feature of *Artemisia* and can be employed as problem-solving character in *Artemisia*. Cladistic revelation based on micromorphology of pollen grains deep-rooted *Seriphidium* as a subgenus of *Artemisia*.

5. The investigations based on the collection data of this study regarding phytogeography of *Artemisia* revealed that majority of (44 %) *Artemisia* species are present in Gilgit district as compared to other districts of Gilgit-Baltistan Pakistan. This study substantiated

that *A. maritima* and *A. sieversiana* are the most plentiful species found in all regions of the studied districts. Moreover, *A. maritima* and *A. herba-alba* are the most dominant in the hills and mountains of different districts of Gilgit-Baltistan region of Pakistan.

6. This study for the first time reports molecular phylogenetic reconstruction of *Artemisia* from the Northeast region (Gilgit-Baltistan) of Pakistan using the nrDNA (ITS and ETS) and cpDNA (*psbA-trnH*) sequences. The obtained results based on molecular data confirmed the polyphyletic appearance of subgenus *Artemisia* and *Absinthium*. All other subgenera including *Seriphidium, Pacifica* and *Dracunculus* were found to be monophyletic. This study observed three new groups of undescribed *Artemisia* taxa from the Northeast region of Pakistan. One observed new group (Group I) belongs to the subgenus *Dracunculus*, and the other two new groups (Group II and III) belongs to the subgenus *Absinthium*. One undescribed taxon of *Artemisia* in group I was found with *A. japonica* and *A. desertorum* lineages. Four undescribed taxa within group II were designated with *A. rutifolia* lineage. Five undescribed taxa within group III were found in the same lineage with *A. sieversiana*.

The BLAST results endorsed that the undescribed taxa of *Artemisia* with in all groups displayed their close connection with subgenera *Absinthium* and *Dracunculus*. Based on the current data and all available in literature, it is concluded that the morphological studies coupled with modern molecular techniques may lead to the clear infrageneric classification of the genus *Artemisia*. It will also clarify and characterize the undescribed taxa reported in this study.

5.9. Recommendations

This primitive awareness of ethnobotany of *Artemisia* from Gilgit-Baltistan region of Pakistan may give fundamental insights for researchers to scrutinize their chemical composition and important biological activities to cope with different health problems particularly in the area of oncology, neurology, hepatology, dermatology and parasitology. Therefore, a wide-ranging ethnobotanical survey of *Artemisia* in all districts of Gilgit-Baltistan region of Pakistan should be accompanied in future for the documentation of more information.

The classical grouping of *Artemisia* has previously raised many objections due to its dependency in floral features. Straightforwardly, the classical grouping is based only on floral morphology of *Artemisia*. For an instance, Kaul and Bakshi, (1984) corroborated that the two sections *Absinthium* and *Artemisia* are different from each other on the basis of only receptacle. Like the receptacle in subgenus *Absinthium* is protected with long hairs while the receptacle in subgenus *Artemisia* is naked.

Gilgit-Baltistan region of Pakistan is a hub of medicinally important plants and the existence of novel *Artemisia* species in this region may provide better consequences in the classifications based on morphology. The morphological revelations of this study validates that more species from all sections of genus *Artemisia* should be extensively investigated from this far flung region with special emphasis on micromorphological attributes in amalgamation with molecular inquiries.

The phylogenetic analysis corroborates that the grouping of all undescribed taxa of *Artemisia* with in the three groups disjointedly from each other specifies the further polyphasic examinations. The morphological studies coupled with modern molecular techniques may lead to the clarification and characterization of the undescribed taxa reported in this study.

Finally, the data from all the taxonomic areas including phytogeography, morphology, palynology, anatomy, phytochemistry, pharmacology, and karyology of the genus *Artemisia* should be combined with molecular data. This approach may elucidate all the existed taxonomic disagreements of the genus *Artemisia*.

CHAPTER 6

6. REFERENCES

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APPENDICES

Appendix 1. Questionnaire used to gather the ethnobotanical information from the native people of Gilgit-Baltistan region of Pakistan.

- Name of the research project/dissertation title: _____
- Botanical Name of plant: _____
- Collectors name:_____
- Habit:_____
- Habitat:_____
- Altitude:_____
- Latitude:_____
- Longitude:_____
- Plant identified by:_____
- Informant name:_____
- Gender:_____Male/Female
- Age:_____
- Occupation:_____
- Ethnic group/cast:_____
- Plant local name:_____
- Location:_____
- District:_____
- Is it familiar by this name: _____ Yes/No
- Other places of plants availibility:_____
- Blooming and Fruiting period:_____
- Folk uses of plant: _____
- Traditionally used by: _____ Pansar, Hakeem etc.
- Any side effects:_____
- Part used: _____Branches, Leaves, Roots, Flowers, Seeds,
- Is it sold in the market: _____ Yes/No
- Per Kg rate: _____
- Is it favorite food of livestock: _____ Yes/ No
- Livestock name:_____Cow, Goat, Sheep,Yak, etc.
- Favourite part:_____ Leaves, Branches, Seeds, Flowers,
- Is it used for cure of human diseases: _____ Yes/No
- If yes then for which disease:_____
- How it is used by local community:_____
- Availibility season:_____ Autumn, Spring, Winter, Summer,
- Local status:_____Endangered, Rare, Common, vulnerable
- Researcher name:_____

Appendix 2. List of specimens included in the phylogenetic analysis with Genbank references. Origin and detail of newly sequenced taxa is given in Table 3.1 and their Genbank references are provided in Table 3.5, 3.6 & 3.7.

INGROUP: *A. absinthium* ITS (Hobbs and Baldwin, 2013) JX051763; ETS (Hobbs and Baldwin, 2013) JX069443; *PsbA-trnH* (Liu *et al.*, 2016) KX581940. *A. afra* ITS (Hobbs and Baldwin, 2013) JX051763; ETS (Hobbs and Baldwin, 2013) JX069431; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073899. *A. annua* ITS (Daniel and Knoess, Unpublished) FJ528301; ETS (Sanz *et al.*, 2008) DQ028879; *PsbA-trnH* (Liu and Ji, 2016) FJ418749. *A.arborescens* ITS (Hobbs and Baldwin, 2013) JX051741; ETS (Hobbs and Baldwin, 2013) JX069429; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073897. *A. argyi* ITS (Doh *et al.*, 2016) KT965666; ETS (Hobbs and Baldwin, 2013) JX069383; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073851. *A. armeniaca* ITS (Hobbs and Baldwin, 2013) JX051693; ETS (Hobbs and Baldwin, 2013) JX069393; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073861. *A. atrata* ITS (Hobbs and Baldwin, 2013) JX051761; ETS (Hobbs and Baldwin, 2013) JX069442; *PsbA-trhH* (Hobbs and Baldwin, 2013) JX073910. *A. australis* ITS (Hobbs and Baldwin, 2013) JX051753; ETS (Hobbs and Baldwin, 2013) JX069435; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073903. *A. austriaca* ITS (Liu *et al.*, 2016) KX581797; ETS (Sanz *et al.*, 2008) DQ028844; *PsbA-trnH* (Liu *et al.*, 2016) KX581950. *A. biennis* ITS (Hayat *et al.*, Unpublished) KC493084; ETS (Hayat *et al.*, Unpublished) JQ844555. *A. brevifolia* ITS (Hayat *et al.*, Unpublished) KC493069; ETS (Hayat *et al.*, Unpublished) JQ844560. *A. californica* ITS (Hobbs and Baldwin, 2013) JX051687; ETS (Hobbs and Baldwin, 2013) JX069388; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073856. *A. campestris* (Hobbs and Baldwin, 2013) JX051736; ETS (Hobbs and Baldwin, 2013) JX069426; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073894. *A. capillaris* ITS (Pellicer *et al.*, Unpublished) JF326535; ETS (Pellicer *et al.*, Unpublished) JF326591; *PsbA-trnH* (Liu and Ji, 2016) FJ418750. *A. chamaemelifolia* ITS (Hobbs and Baldwin, 2013) JX051730; ETS (Hobb and Baldwin, 2013) JX069420; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073888. *A. chinensis* ITS (Yao *et al.*, 2017) MF063751; ETS (Hobbs and Baldwin, 2013) JX069438; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073906. *A. commutata* ITS (Pellicer *et al.*, Unpublished) JF326538; ETS (Pellicer *et al.*, Unpublished) JF326594. *A.*

desertorum ITS (Hayat *et al.*, Unpublished) KC493082; ETS (Pellicer *et al.*, Unpublished) JF326597. *A. douglasiana* ITS (Hobbs and Baldwin, 2013) JX051723; ETS (Hobbs and Baldwin, 2013) JX069417; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073885. *A. dracunculus* ITS (Pellicer *et al.*, Unpublished) JF326545; ETS (Pellicer *et al.*, Unpublished) JF326601; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073881. *A. dubia* ITS (Hayat *et al.*, Unpublished) KC493081; ETS (Hayat *et al.*, Unpublished) JQ844556. *A. giraldii* ITS (Pellicer *et al.*, Unpublished) JF326549; ETS (Pellicer *et al.*, Unpublished) JF326605. *A. glomerata* ITS (Hobbs and Baldwin, 2013) JX051738; ETS (Hobbs and Baldwin, 2013) JX069427; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073895. *A. gmelinii* ITS (Chen and Han, Unpublished) FJ980317; ETS (Tkach *et al.*, 2008) AM397982; *PsbA-trhH* (Liu *et al.*, 2016) KX581959. *A. herbaalba* ITS (Hayat *et al.*, Unpublished) KC493080; ETS (Hayat *et al.*, Unpublished) JQ844563; *PsbA-trnH. A. indica* ITS (Hobbs and Baldwin, 2013) JX051676; ETS (Hobbs and Baldwin, 2013) JX069379; *PsbA-trnH* (Chen *et al.*, Unpublished) KU555810. *A. japonica* ITS (Hayat *et al.*, Unpublished) KC493078; ETS (Hayat *et al.*, Unpublished) JQ844553; *PsbA-trnH* (Rashmi *et al.*, 2015) KP885710. *A. kauaiensis* ITS (Hobbs and Baldwin, 2013) JX051754; ETS (Hobbs and Baldwin, 2013) JX069436; *PsbA-trnH* (Hobbs and Baldwin, 2013) JX073904. *A. kitadakensis* ITS (Hobbs and Baldwin, 2013) JX051732; ETS (Hobbs and Baldwin, 2013) JX069465. *A. kurramensis* ITS (Hayat *et al.*, Unpublished) KC493068; ETS (Hobbs and Baldwin, 2013) JX069469. *A. maritima* ITS (Hayat *et al.*, Unpublished) ITS KC493077; ETS (Hayat *et al.*, Unpublished) JQ844558. *A. mauiensis* (Hobbs and Baldwin, 2013) JX051755; ETS (Hobbs and Baldwin, 2013) JX069437; *PsbA-trhH* (Hobbs and Baldwin, 2013) JX073905. *A. monosperma* ITS (Pellicer *et al.*, Unpublished) JF326565; ETS (Pellicer *et al.*, Unpublished) JF326622. *A. montana* ITS (Hobbs and Baldwin, 2013) JX051677; ETS (Hobbs and Baldwin, 2013) JX069380; *PsbA-trnH* (Riggins and Seigler, 2012) JN862061. *A. princeps* ITS (Doh *et al.*, 2016) KT965667; ETS (Hobbs and Baldwin, 2013) JX069462; *PsbA-trnH* (Chen *et al.*, Unpublished) KU855307. *A. roxburghiana* ITS (Hayat *et al.*, Unpublished) KC493076; ETS (Hobbs and Baldwin, 2013) JX069384; *PsbA-trhH* (Gandhi *et al.*, 2014) KJ372429. *A. rupestris* ITS (Song *et al.*, 2013) KF724979; ETS (Tkach *et al.*, 2008) AM398013; *PsbA-trnH* (Peterson and Peterson, 2016) AJ401617. *A. rutifolia* ITS (Hobbs and Baldwin, 2013) JX051672; ETS (Sanz *et al.*, 2008) DQ028849;

Appendix 3. DNeasy® kit (QIAGEN) method; Source: www.qiagen.com/literature

Bench Protocol: DNeasy Plant Mini

QIAGEN

Note: Before using this bench protocol, you should be completely familiar with the safety information and detailed protocols in the *DNeasy Plant Handbook*.

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If necessary, redissolve any precipitates in Buffers AP1 and AP3/E concentrate.
- Ensure that ethanol has been added to Buffers AW and AP3/E.
- Preheat a water bath or heating block to 65°C.

Procedure

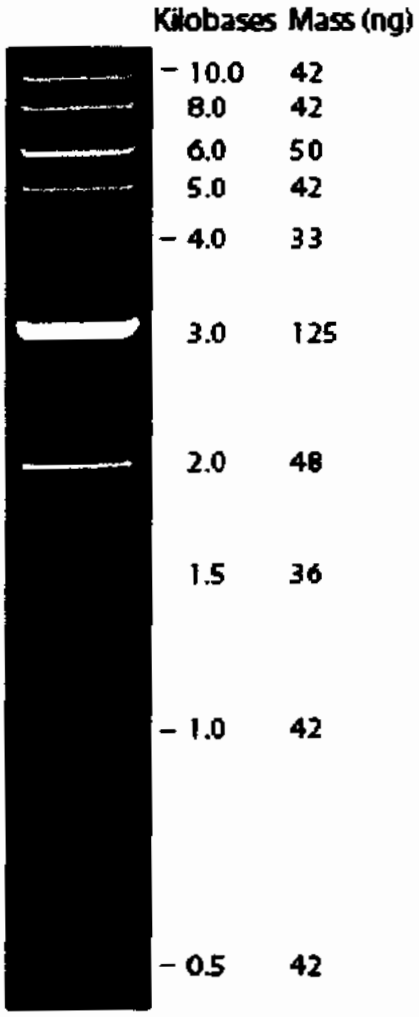
1. **Disrupt the sample material (≤100 mg wet weight or ≤20 mg lyophilized tissue) using the TissueRuptor, the TissueLyser, or a mortar and pestle.**
2. **Add 400 µl Buffer AP1 and 4 µl RNase A. Vortex and incubate for 10 min at 65°C. Invert tube 2–3 times during incubation.**

   Note: Do not mix Buffer AP1 and RNase A before use.
3. **Add 130 µl Buffer AP2. Mix and incubate for 5 min on ice.**

   Recommended: Centrifuge the lysate for 5 min at 20,000 x *g* (14,000 rpm).
4. **Pipet the lysate into a QIAshredder Mini spin column in a 2 ml collection tube. Centrifuge for 2 min at 20,000 x *g* (14,000 rpm).**
5. **Transfer the flow-through fraction into a new tube without disturbing the pellet. Add 1.5 volumes of Buffer AP3/E, and mix by pipetting.**
6. **Transfer 650 µl of the mixture into a DNeasy Mini spin column in a 2 ml collection tube. Centrifuge for 1 min at ≥6000 x *g* (≥8000 rpm). Discard flow-through. Repeat this step with the remaining sample.**
7. **Place the spin column into a new 2 ml collection tube. Add 500 µl Buffer AW, and centrifuge for 1 min at ≥6000 x *g*. Discard flow-through.**
8. **Add another 500 µl Buffer AW. Centrifuge for 2 min at 20,000 x *g*.**

   Note: Remove the spin column from the collection tube carefully so the column does not come into contact with the flow-through.
9. **Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube, and add 100 µl Buffer AE for elution. Incubate for 5 min at room temperature. Centrifuge for 1 min at ≥6000 x *g*. Repeat this step.**

Appendix 4. 1kb standard size DNA ladder (500bp to 1kb) (N-3232L, Biolabs Company) for the determination of amplified DNA size



Appendix 5. QIAquick Gel Extraction Kit (QIAGEN) method; Source: www.qiagen.com/literature

Quick-Start Protocol

QIAquick® Gel Extraction Kit

The QIAquick Gel Extraction Kit (cat. nos. 28704 and 28706) can be stored at room temperature (15–25°C) for up to 12 month

Further information

- *QIAquick Spin Handbook*: www.qiagen.com/HB-1196
- Safety Data Sheets: www.qiagen.com/safety
- Technical assistance: support@qiagen.com

Notes before starting

- This protocol is for the purification of up to 10 µg DNA (70 bp to 10 kb)
- The yellow color of Buffer QG indicates a pH ≤7.5. DNA adsorption to the membrane is only efficient at pH ≤7.5
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume)
- Isopropanol (100%) and a heating block or water bath at 50°C are required
- All centrifugation steps are carried out at 17,900 × *g* (13,000 rpm) in a conventional tabletop microcentrifuge

1 Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

2 Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~ 100 µl). The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, add 6 volumes Buffer QG.

3 Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The mixture turns yellow.

4 Add 1 gel volume isopropanol to the sample and mix.

5 Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 µl, load and spin/apply vacuum again.

6 If DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 500 µl Buffer QG to the QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.

7 To wash, add 750 µl Buffer PE to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.

Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt-ended ligation), let the column stand 2–5 min after addition of Buffer PE.

Centrifuge the QIAquick column in the provided 2 ml collection tube for 1 min to remove residual wash buffer.

8 Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

9 To elute DNA, add 50 µl Buffer EB (10 mM Tris-Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.

10 If purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

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