Pollination Biology of Mandarin Orange (*Citrus reticulata* Blanco) in the Darjeeling and Sikkim Himalayas

A Thesis Submitted

To

Sikkim University

In Partial Fulfilment of the Requirements for the

Degree of Doctor of Philosophy

By

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December 2021

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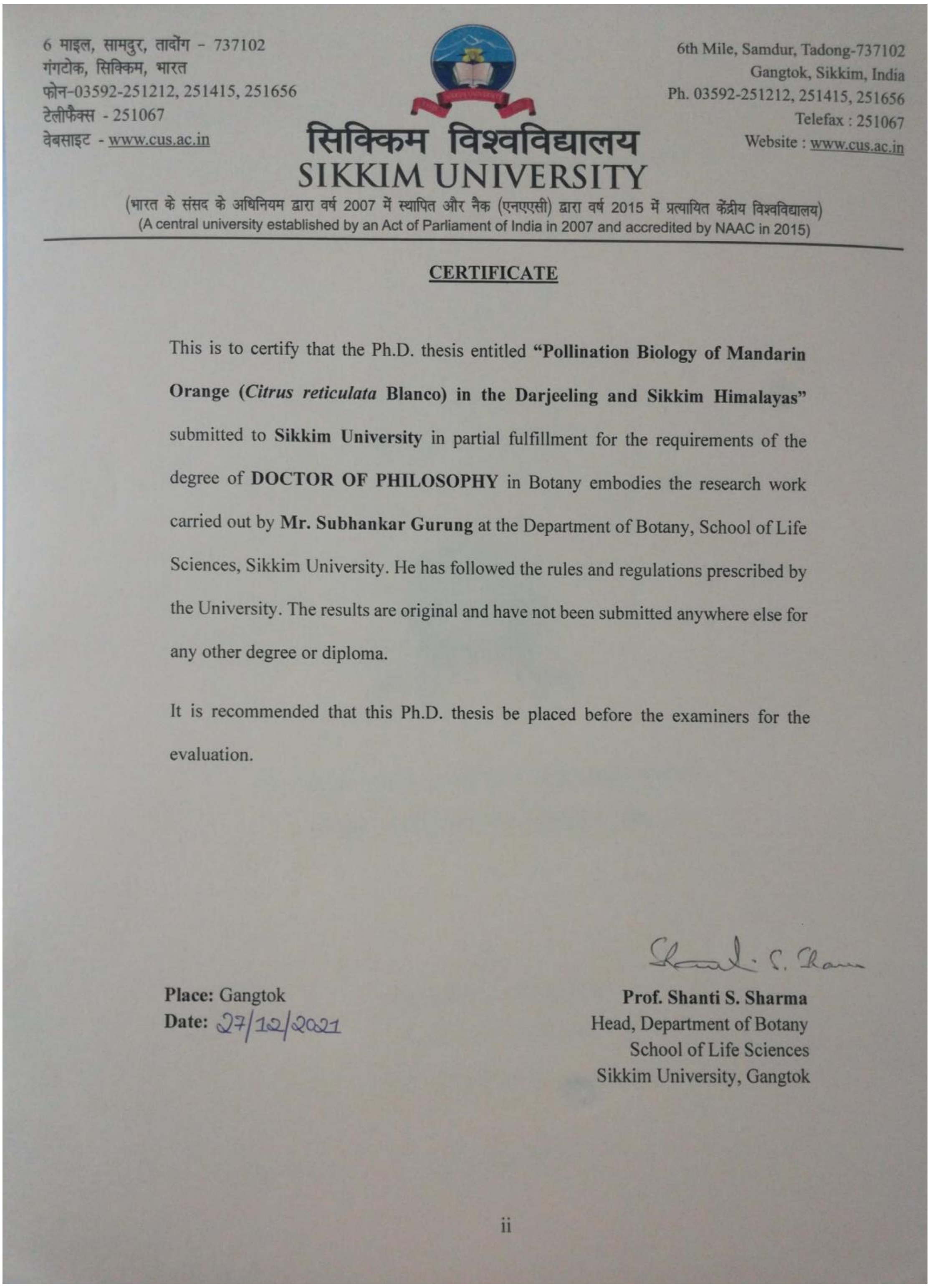

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DECLARATION

I declare that the Ph.D thesis entitled **"Pollination Biology of Mandarin Orange (*Citrus reticulata* Blanco) in Darjeeling and Sikkim Himalayas"** submitted for the degree of Doctor of Philosophy to Sikkim University, is an original work, conducted under the supervision of my supervisor **Dr. Arun Chettri,** Assistant Professor, Department of Botany, Sikkim University.

I also wish to apprise that the thesis does not bear content that has been submitted for a degree of examination at any university or institution. Further, the references used to augment the research and the materials obtained have been duly acknowledged.

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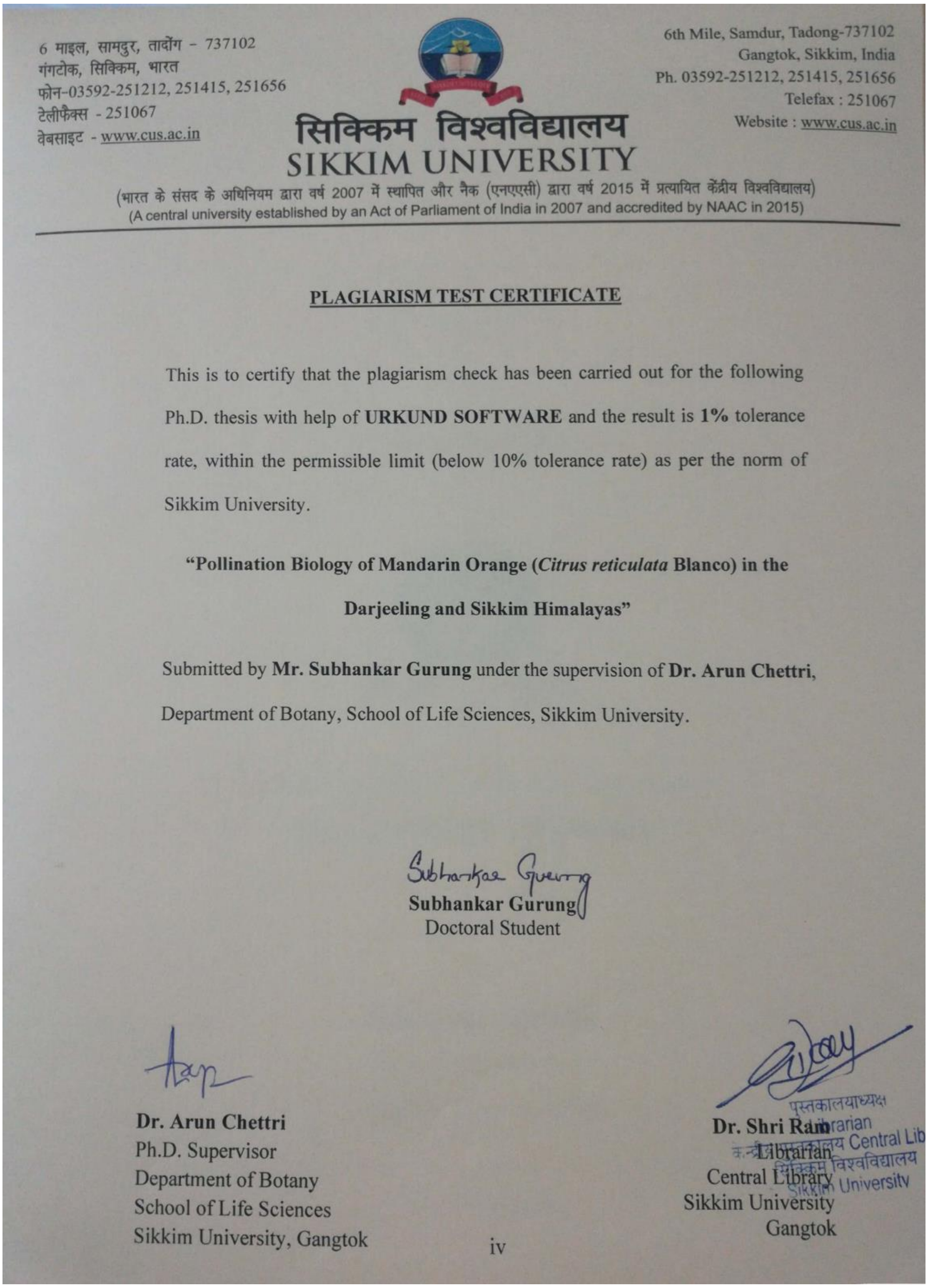

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"Pollination Biology of Mandarin Orange (*Citrus reticulata* Blanco) in the Darjeeling and Sikkim Himalayas"

Submitted by **Mr. Subhankar Gurung** under the supervision of **Dr. Arun Chettri**, Department of Botany, School of Life Sciences, Sikkim University.

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Acknowledgments

My *baba* had a bizarre habit of plucking some herbs while he walked his way to school or at home. I still remember the younger version of him holding an herb plucked right from its roots as if it is an episode in my life that occurred recently. I stood confused but lacked the curiosity to ask him why he did it frequently. It was later in my life that I realized that my *baba* was a Botanist and the herbs that he plucked were nothing but a part of a curriculum to be taught to his pupils in school. I now understand that our fate was entwined and believe that I was always destined to finish what he had started, but before I get there, I must acknowledge every person who paved this way for me.

I believe my journey to become a Ph.D. scholar initiated the day I joined the Department of Botany, Sikkim University in the year 2013. The opportunities and the exposure that the Department provided helped me grow and build a strong foundation to pursue my career in research. I owe the Department and the faculties thanks for all of the seminars that were held, which provided a cordial platform for interaction with renowned scientists and fellow researchers, allowing me to gain a greater understanding of the subject. Firstly, I thank the Head of the Department of Botany Prof. Shanti S. Sharma for his constant support and encouragement. I am grateful to Dr. N Sathyanarayana, Dean of School of Life Sciences and former Head of the Department of Botany, and Dr. Dhani Raj Chhetri, former head of the Department of Botany and Dean of Students Welfare for letting me host some of the major events that the Department organized. Such opportunities have allowed me to push the envelope and strive for better. Dr. Dhani Raj Chhetri once said to me *"if you are not growing, you are just waiting for death"* - it is such teachings that have become tenets of my life and have compelled me to always strive for growth. I am thankful to Dr.

N. Bijayalaxmi Devi for instilling an interest in ecology in me. I am grateful to Dr. Santosh Kumar Rai and Dr. Arun Kumar Rai for being amiable towards me throughout. I take this opportunity to thank Dr. Santosh Kumar Rai for considering mean able candidate to work in a project funded by ICIMOD. It was under his guidance that I received an opportunity of a lifetime to participate and represent the Department and Sikkim University in Asia Pacific Youth Forum (APYF 2015) organized by ICIMOD in Kathmandu, Nepal. At an age when most of the students are confused about their careers, I experienced an epiphany about stepping into the world of research because of the continuous interactions I had with my seniors. I will forever be indebted to Aita Rani Subba, Dr. Aditya Pradhan, Dr. Nima Tshering Lepcha, Dr. Pankaj Tripathi, Dr. Ranjith Kumar Pittala, Dr. Dawa Lhendup Lepcha, Dr. Pribat Rai for the influence, support, and encouragement I have garnered from them throughout the journey. I am grateful to Prem Prakash Singh, Research Scholar of NEHU, and Dr. Harsh Singh, Research Associate NBRI, Lucknow for their guidance and encouragement. Wishing to be a Ph.D. scholar can send jitters of excitement down one's spine but can be equally daunting when one steps into it. I am grateful to Aditya da for helping me take the initial steps in this voyage and making sure that I don't fumble. It is evident that a journey of a Ph.D. student can be filled with upheavals of emotional swings, which can take a toll on them at times. Such emotions were poured in discourse with friends, along with a cup of tea in Roshan das' canteen. I am grateful to Aita di, Pooja di for their love, concern, and moral support in times of dire need. I would also like to thank my batchmates Dr. Patrush, Raksha, Alice, and Ashis for being alongside me since the day we embarked on this journey together back in 2015.

It is indeed true that every event that occurs in one's life has a purpose and every person one meets is for a reason. I take this opportunity to acknowledge an important role ATREE has played in my life. I thank Dr. Sarala Khaling, Regional Director (ATREE), Dr. Soubadra Devy, FELLOW (ATREE) for allowing me to intern in one of their projects related to ecosystem services. It is there that I garnered a basic insight on pollination which I later incorporated in my Ph.D. program. I would also like to remember Vikram Pradhan, Dr. Rakesh Basnet, Andrew for helping me acquaint myself with pollinators and pollination studies. I am also grateful to Karuna di for introducing me to ATREE and making me realize the importance of being bold and independent.

Above all, I am grateful to Dr. Arun Chettri for noticing the potential in me and allowing me to explore the avenues of research under his supervision. I thank him for allowing me to develop my insights and for the freedom to explore anything that drew my attention. This can be reflected in the variety of research articles we have been able to publish over a span of five years. I thank him once again for considering me able to work as a Junior Research Fellow (JRF) in one of his projects which helped me amass experience and sustain myself in an expensive city like Gangtok. Getting an opportunity to work under him has helped me a lot to groom myself as a researcher. I would also like to sincerely thank Mrs. Anjila Tamang for her support, concern, and encouragement.

I take this opportunity to thank the donors – JICA assisted Sikkim Biodiversity Conservation and Forest Management Project (SBFP), Forest Environment & Wildlife Management Department, Government of Sikkim for their generous funding for the project *"Study of Oak Forest ecosystem in Sikkim Himalaya with special reference to climate change, plant species diversity and distribution"*. My involvement in the project allowed me to work with budding researchers from The Mountain Institute – Prakash Chettri, Mahindra Luitel, and Kalzang Da. I am glad that we could share a great camaraderie in both personal and professional life. I am grateful to have met eminent researchers - Dr. Bharat Kumar Pradhan, Dr. Ghanashyam Bastola who were associated directly or indirectly with the projects that we were handling. I am also thankful to the non-teaching staff for their cooperation in all the financial and technical help that the project required. I am also indebted to Bondhu da of Hilley and Thakur da of Lingee-Payong for assisting me in the field.

Some instances have helped me immensely in the completion of my work. I thank Dr. N. Sathyanarayana for his suggestion to seek help of Dr. Shivanna for pollination studies. Fortunately, my quest for knowledge led me to his book, a pollination manual that helped me immensely in understanding, designing, and completing pollination experiments in the orchards. I am grateful to Aditya da for helping me design my research and assisting me in my study that required GIS intervention. I thank Dr. Jiwan, Dr. Sailendra, Dr. Jins for their insight into statistics that I believe has helped me enormously in enhancing the quality of my research. I thank Dr. Sujata, Prof. Dr. J.P. Tamang, Dr. Buddhiman Tamang for their academic guidance during the entire period of my course work. I am grateful and will forever be indebted to Dr. David Lalasama Biate, Scientist-C, Botanical Survey of India, Eastern Regional Centre, Shillong for being generous enough to help me with the SEM analysis of my pollen samples.

I take this opportunity to thank the Department of Botany, Sikkim University for facilitating this research. I thank N.B (Andrew) Subba, Deepak Chettri, and Roshan Rai for being generous and kind in providing laboratory facilities. Although I was trying hard to understand my work and seeking help from respective experts, I admit that I was a bit reluctant in the early days to initiate my research. One of the major reasons for my reluctance was fear and lack of confidence. At such trying times, Ankit came all the way from Jalpaiguri to accompany me for my field visits. I'm blessed to have a friend like him and I thank him for his unconditional love and support. I thank Mamta and Meera who were pursuing their Masters then for helping me with the laboratory chores. I thank Alice and Aita di for accompanying me to the orchards located in the far-flung areas of Sikkim. I would like to thank all the farmers of Darjeeling and Sikkim for their cooperation and generosity. I would like to thank Pribat da, Aita di for accompanying me to Dzongu where I performed a portion of my pollination studies. We look back to the ride to the North with equal vigor and nostalgia.

I take this opportunity to thank Dr. Partha Sarathi Ghose, Ms. Priyadarshini Shrestha, Ms. Lak Tsheden Theeng of World-Wide Fund (Sikkim) for allowing me and my peers to assist them in a project which took me for the first time to North Sikkim. It is there that I had an opportunity to meet Mr. Tenzing Gyatsho Lepcha. I shall always remain indebted to Tenzing Anum and his family for allowing me to conduct my study in their orchard. I am grateful for the generosity he bestowed while I fiddled with innumerable flowers and kept a close watch on my set-up while I was away. Similarly, I am grateful to my *kaka* who generously allowed me to conduct my experiments in his orchard in Darjeeling.

The journey was made easy and less tiresome with the presence of amiable fellow researchers and juniors in the Department. I take this opportunity to thank Bhupen Chettri, Abhijeet Chhetri, Poonam Chetry, Priyanka Kumari, Binu Gogoi, Bikram Subba, Suman Sir, Say Say Hang, Tshering for their immeasurable moral support. I am thankful to the staff of the library for providing an environment congenial for ideas to generate. A lot of my research has been a result of all the sole contemplation I have done in the library. I am grateful to all the non-teaching staff who have known me personally for being such endearing towards me.

I take this opportunity to thank Nirnay and Vivek for being such good friends throughout. I fondly remember Nitesh Lohagan, Nitesh Lohar, and the time we spent together. I will forever be indebted to Keshav Bishunkey, Lab Assistant of the Department of Computer Applications for being there for me during my rough days. I have and continue to learn a lot from him.

My heartfelt gratitude to my friends back home who rooted for me. I am grateful to my family for their endless support. They consider me formidable and I believe it is their confidence in me that has made this journey possible. I am grateful to my brother Nirpen da for being so proud of me that I had no other option than to make it. Sonam *bhawju* and Bhanu for the financial aid during 105 *bandh* in Darjeeling – I am highly indebted to them for their generosity. I thank *Maili kaki*, Sakila *Phupu-Phupa*, *Kanchi Kaki-Kaka*, Shikha *bhawju*, Pema *bhawju*, Anjana *bhawju* for welcoming me with a bright smile every time I returned home for a retreat. My *boju* who loved me dearly – guide me from above. Big thanks to my cousins - Binam, Nigam da, Sangam da, Saroj da, Sarika di, Ashika di, Sulu, Neha, Subnam, Supriya, Unam di. I partly dedicate this thesis to my nieces and nephew – Janvi, Prachi, Ariana, Arnav (Dallu), Adishree. Rajeena *Phupu* as a guardian took care of me and made sure I studied during my school days. I believe it is her dedication that has made me capable enough to pursue Ph.D. I will forever be indebted to her for everything she has done for me.

Lastly, I thank and dedicate this thesis to *Baba-Aama* – they have been the biggest source of inspiration to me right from the beginning. It is their relentless support that has given me the zeal to complete the thesis. Steve Jobs once said, *"You can't connect the dots looking forward, you can only connect them looking backward. So, you have to trust the dots will somehow connect in your future"* – my life has never been clearer as it is now. As I mentioned earlier, my *baba* did his Master's in Botany way back in the 1980s and had a bright future ahead of him. Unfortunately, due to family obligations and unprecedented events in his life, he could not pursue Ph.D. and had to settle for what life offered him. I could sense his regret but never contemplated how I could help him get over it. I did not intend to be a Botanist; I would have rather settled for some corporate job in Bangalore where I completed my Bachelor's. I had no intention of coming back. It was due to some personal tragedy that I was pulled back and the rest is history. The biggest satisfaction I have right now is my father saying, *"You accomplished what I could not".* My story is no different than a typical middle-class son's story aspiring to fulfil his parents' unfulfilled dream. The wait was long, roads at times bumpy but I did this for you *Baba*. I hope you are proud of me.

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Abbreviations

BC	Before Christ
ICIMOD	International Centre for Integrated Mountain Development
UNCTAD	United Nations Conference on Trade and Development
USDA	United States Department of Agriculture
SOD	Superoxide dismutase
NEH	North-Eastern Hill Regions
QTL	Quantitative trait locus
AFLP	Amplified fragment length polymorphism
DNA	Deoxyribonucleic Acid
RAPD	Random Amplified Polymorphic DNA
ISSR	Inter Simple Sequence Repeats
GIS	Geographic Information System
GPS	Global Positioning System
SAPCC	Sikkim Action Plan on Climate Change
DSR	District Survey Report
NHB	National Horticultural Board
IPGRI	International Plant Genetic Resource Institute
ANOVA	Analysis of Variance
MANOVA	Multiple Analysis of Variance
PCA	Principal Component Analysis
PC	Principal Component
TSS	Total Soluble Solids
SEM	Scanning Electron Microscope
SPSS	Statistical Package for the Social Sciences
HSD	Honestly Significant Difference
UV	Ultra Violet
BSA	Bovine Serum Albumin
RH	Relative Humidity
ROS	Reactive Oxygen Species
AD	Anno Domini

Symbols and Units

ha	Hectare
h	Hour
%	Percentage
µm	Micro meter
µM	Micromolar
ml	Millilitre
µl	Microlitre
g	Gram
l	Litre
nm	Nano meter
N	Normal
mg	Milligram
°C	Degree Celsius
CO_2	Carbon-dioxide

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CHAPTER 1

GENERAL INTRODUCTION

1.1 History and origin of Citrus

The origin of *Citrus* is associated with a history filled with controversy and some legendary anecdotes (Liu et al. 2012). It is widely believed that *Citrus* originated in certain parts of Southeast Asia including China, India, and Malay Archipelago (Bartholomew and Sinclair 1952; Gmitter and Hu 1990). However, recent research suggests that the true origin of *Citrus* is Australia, New Caledonia (off eastern Australia), and New Guinea (Anitei 2007). However, detailed molecular analysis (Pfeil and Crisp 2008) and the whole-genome phylogenetic analysis refute the theory of the Australian origin of *Citrus* (Beattie et al. 2008). In India, *Citrus* is mentioned in a collection of Hindu sacred books which dates prior to 800 BC, where lemons and citron are named as *jambila* (Ramana et al. 1981). Further, *Citrus*, particularly citron was associated with the elephant-headed God, *Ganesh* and therefore considered sacred (Viglietti 2016). In China, the oldest references of *Citrus* were made in the book "*Tribute of Yu"* during the reign of Ta Yu (2205 – 2197 BC) (Liu et al. 2012). It was during this period that *Citrus* fruits such as mandarins and pummelos were presented to the imperial courts to revere the emperor (Webber 1967; Scora 1975; Nagy and Attaway 1980). According to 5th century Chinese manuscripts "Shu Ching", *Citrus* names such as *chu* (kumquats and mandarins), *yu* (pummelo, *Citrus maxima*), *yuzu* (*Citrus junos*), and *chih* (*Poncirus trifoliata*) appears for the first time (Viglietti 2016). Further, citron is described for the first time in a book entitled *Plants of the southeast region of Chi-Han* in the year 304 CE (Inglese and Sortino 2019). In a three-volume monograph *Chü lu,* Han Yen-Chih in 1178 AD described 27 varieties of *Citrus* cultivated during the reign of Emperor Ta Yu (Wu et al. 2018; Inglese and Sortino 2019). An ancient Chinese cultivar *Chu Sha Kat* which consists of a pure *Citrus reticulata* genome is supposedly described in the former monograph. It was from India and China that the *Citrus* varieties, particularly citron reached the near east and the Mediterranean (Nicolosi et al. 2005). Theophrastus at about 310 BC described citron as the apple from the land of Medes and recognized its potential as an antidote (Inglese and Sortino 2019). In addition to its medical properties, it became an important part of the Jewish feast of Tabernacles (Scora 1975). Through the Jewish communities, the fruit was traded over the entire Mediterranean region. *Citrus* fruits spread throughout Europe through the rise of Islam and the Arab Empire (Scora 1975). It is during this era that other *Citrus* fruits such as lemon and sour orange reached the Mediterranean (Nicolosi et al. 2005). However, while citron was already known in the Roman Empire there was ambiguity regarding the introduction of lemon to the Roman Empire (Inglese and Sortino 2019). Although a different report suggests that Romans originally identified citron, lemon, and lime under the terminology of "yellow skin *Citrus*".

The Portuguese were the first to bring sweet oranges from India (Scora 1975) and China to Europe (Inglese and Sortino 2019). A new age began in the 16th century when sweet oranges garnered the status of commercial prominence (Scora 1975). From Europe *Citrus* were brought to the New World by Christopher Columbus in his second journey (Inglese and Sortino 2019). The first orange trees were grown in Mexico in 1518 while the Jesuits in the last third of the 16th Century were instrumental in spreading *Citrus* fruits across South America (Inglese and Sortino 2019). *Citrus* was introduced to Brazil between 1530 and 1540 (Inglese and Sortino 2019). Further, *Citrus* was introduced to Florida by Spanish settlers in 1565, particularly in St. Augustine (Scora 1975). *Citrus* fruits, citric acid, and essential oils were in high demand in the 18th century, therefore new varieties for commercial edible fruits were produced quickly (Inglese and Sortino 2019). The hybrid of pummelo and sweet orange, grapefruit (*Citrus paradisi* Macf.) was produced for commercial intention around the end of 18th century (Viglietti 2016). Hitherto, the cultivation of *Citrus* reached eastern Spain, Sicily, and Calabria. It was no sooner that mandarin became the most popular fruit which was cultivated and sold worldwide (Viglietti 2016). *Citrus* is now grown in over 140 nations throughout the world, with cultivation and production concentrated in the Northern Hemisphere's key regions (UNCTAD 2004; Fig. 1).

Fig. 1. The world’s major producing regions for *Citrus* fruits (highlighted in orange). (Adapted according to FAO data from UNCTAD 2004).

1.2 Genealogy of cultivated Citrus

The genus *Citrus* belongs to the angiosperm subfamily Aurantioideae of the Rutaceae family (Wu et al. 2018). *Citrus* crop is mostly produced in the tropical and subtropical areas on either side of the belt around the equator, with cultivation and production being prominent in the Northern Hemisphere (Ramana et al. 1981; UNCTAD 2004, Liu et al.

2012). *Citrus* is mainly grown in North America, Central America, Mediterranean, and East Asia in the Northern Hemisphere and South America, South Africa, and Australia in the Southern Hemisphere (Ramana et al. 1981). *Citrus* is one of the world's major fruit crops which is appreciated worldwide and contributes largely to human diet (Liu et al. 2012). However, their origins have been unknown, ambiguous, and have been controversial over the past centuries (Talon et al. 2020). A major impediment in understanding its origin is the lack of understanding of the genealogy of complex mixture in cultivated *Citrus* (Wu et al. 2014). *Citrus* are subjected to frequent mutation, polyembryony, and inter and intrageneric hybridization (Ramana et al. 1981). Further, some *Citrus* have propagated apomictically through nucellar embryo (Wang et al. 2017) or grafted (Mudge et al. 2009) which have resulted in the formation of hybrids (Nicolosi et al. 2005). Scora (1975) hypothesised two areas of origin for *Citrus*, tropical and subtropical for the subgenera Papeda and *Citrus*, respectively, based on morphological, genetic, and phytochemical evidence. Furthermore, based on morphological and biochemical characteristics three ancestral species *Citrus medica* (citrons), *Citrus maxima* (pummelos), and *C. reticulata* (mandarins) were identified and are now supported by genetic studies (Barkley et al. 2006; Nicolosi et al. 2000; Wu et al. 2014). Following the premise, various *Citrus* phylogenies have been investigated using a variety of methodologies, including DNA polymorphism analysis (Barret and Rhodes 1976; Nicolosi et al. 2000; Ollitrault et al. 2003; Barkley et al. 2006; Garcia-Lor et al. 2012; Curk et al. 2016) and more recently genome sequencing (Wu et al. 2014, 2018; Ahmed et al. 2019). Wu et al (2018) identified the same three species as ‘fundamental’ *Citrus* species from where commercially important varieties such as lemons, limes, oranges, and grapefruits as hybrids were derived (Wu et al. 2018; Luro et al. 2019).

Nicolosi et al (2000) correctly identified the parentage of several commercial *Citrus* kinds, which was a significant breakthrough. According to their findings based on cpDNA data, citron acted as a male parent in the formation of *Citrus* species. Their research revealed that lemon, bergamot, and Volkamer lemon are citron and sour orange hybrids, Mexican lime is a hybrid of *C. micrantha* and citron, and sour oranges and grapefruit have the same origin. Rough lemon and Rangpur lime were hybrids of citron and mandarin (Deng et al. 1996), whereas Clementine was a hybrid of mandarin and sweet orange (Deng et al. 1996) (Fig. 2).

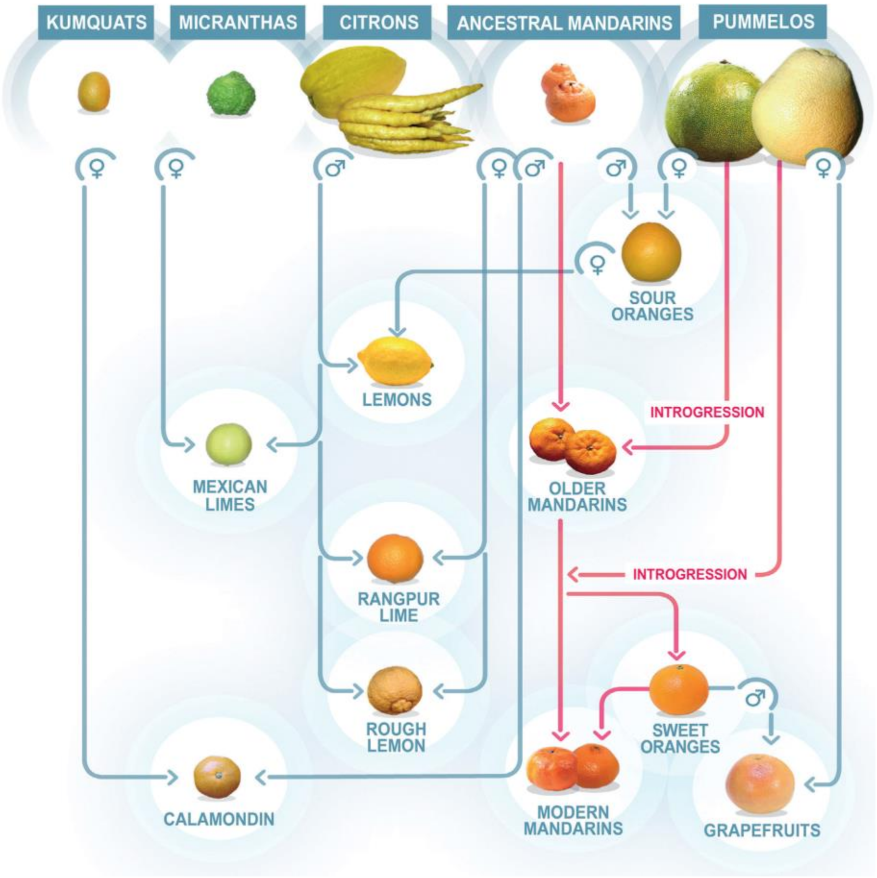


Fig. 2. Genealogy of major *Citrus* genotypes (Source: Talon et al. 2020)

Wu et al (2014) revealed that mandarins are introgressions of *C. maxima* (pummelo) into the ancestral mandarin species *C. reticulata*. Phased pummelo haplotype classified mandarins into three types (Wu et al. 2018). Type 1 mandarin includes pure *C.*

reticulata genomes such as Sun Chu Cha Kat and Tachibana. Type 2 mandarin contained a small amount of pummelo admixture (1%-10%) and only two pummelo haplotypes while type 3 contained higher proportions of pummelo alleles (12-38%) (Wu et al. 2018; Talon et al. 2020). Wu et al (2018) also discovered an enormous network of relatedness between mandarins and sweet oranges, a relationship that appears to be one of the key axioms of edible *Citrus* domestication.

1.3 Taxonomy of Citrus

As discussed above (Section 1.1), the origin and distribution of *Citrus* began in Southeast Asia and spread globally. The cultivated varieties are propagated by grafting and through asexual seed production (apomixis via nucellar embryony) to maintain desirable traits (Wu et al. 2014). Facultative apomixis in the genus prevents the exchange of genes and leads to reproductive isolation (Moore 2001). Such modes of clonal propagation, natural hybridization, high frequency of bud mutation along with long history of cultivation and wide distribution have led to the chaotic *Citrus* taxonomy (Nicolosi et al. 2000). Further, *Citrus* species comprises a wide degree of variation with abundant natural hybridization which has resulted in one species with many subspecies (Moore 2001). There are evidences that suggest some of the wild, true-breeding species are of hybrid origin (Swingle and Reece 1967). In the past, taxonomic classification was prepared based on morphological characteristics or geographical distribution (Nicolosi et al. 2000). However, several characteristics of *Citrus* subgenus made taxonomic classification based on morphology and geography complicated (Moore 2001). Swingle (1943) included 16 species in *Citrus* while Tanaka (1954) defined 147 different species (Nicolosi et al. 2000). Later, phylogenetic analysis by Barrett and Rhodes (1976) identified *C. medica*, *C. reticulata*, and *C. grandis* as true species from where other hybrids were derived (See Section 1.1). Based on the sequence analysis by Shimizu et al (2016), Wu et al (2018) proposed Tachibana to be classified as a subspecies of *C.* reticulata. Wu et al (2018) also argued that the *Citrus* clade contains many genera. Furthermore, based on sequence divergence and whole genome phylogeny, the related genus *Poncirus*, which has been a source of debate since it was first recommended to be included in the genus *Citrus* (Bayer et al. 2009; Nesom 2014), is clearly a different clade that is independent of *Citrus*. Scora (1988) also recognised *C. halimii* as a distinct species. Given the complexity of *Citrus* taxonomy, it is inevitable that as new discoveries are made, present taxonomic beliefs will be questioned, resulting in a reformulation of the genus *Citrus*.

1.4 Citrus in India: Tracing India's Citrus wealth

It is widely agreed that *Citrus* originated and radiated from subtropical and tropical areas of Asia, including India (Bartholomew and Sinclair 1952; Ramana et al. 1981) in the late Miocene (Wu et al. 2018) when a pronounced climate transition from wet to drier condition was witnessed (Clift et al. 2014). Due to its astounding diversity in *Citrus* genetic resources, India occupies a unique position in the world's "*Citrus belt*" (Malik et al. 2013). Various *Citrus* varieties such as *C. medica, C. aurantiifolia*, *Citrus limetta*, *C. reticulata* have been mentioned in Ayurvedic works of Dhanwantri (10th BC), Charak, and Susruta (600 – 500 BC) (Sharma et al. 2004). Later research listed 30 *Citrus* species and botanical varieties that are indigenous and domesticated in India (Singh and Chadha 1993). Bhattacharya and Dutta (1956) reported 17 species, 45 horticultural varieties, and 5 hybrids of *Citrus* in Northeast India. However, Malik et al (2012) reported 15 species to be confined to Northeast India, while 9 species were found in South India, 6 species in Northwestern India, 1 species in Central India, and 9 species throughout India. Apart from the cultivated commercially important species, Indian wild orange, Khasi papeda, Ichang papeda, and Melanesian papeda were reported from the wild or semi-wild state in Northeast India (Brandis 1874; Tanaka 1937; Sharma et al. 2004; Malik et al. 2006). Bhattacharya and Dutta (1956) also reported the occurrence of plants in the deep forests undisturbed by humans. Therefore, considering the diversity of *Citrus* and its occurrence in deep forests, Sharma et al (2004) concluded by assuming Northeast India to be a native home of *Citrus* species. The authors further stated Northeast India to be a treasure house of *Citrus* germplasm.

1.5 Citrus in Darjeeling and Sikkim Himalayas

Present disclosure reveals Northeastern India as one of the centres of origin and diversity of *Citrus* (Tanaka 1954; Scora 1988; Gmitter and Hu 1990). Among the various *Citrus* species, *C. reticulata* (Sikkim mandarin or Darjeeling mandarin) is commercially cultivated and is the most important cash crop of Darjeeling and Sikkim Himalayas. Apart from Sikkim mandarin, studies have been conducted on *C. maxima* and *C. jambhiri* in Sikkim Himalaya (Pradhan et al. 2019). Additionally, *C. medica* and its ethnomedicinal value in Kalimpong have been elaborated by Bhujel et al (2018) and the availability of *C. grandis* in Darjeeling is evident by the studies carried out by Ghosh et al (2014) to test its resistance against *Citrus tristeza* virus and aphid feeding. Besides studies focusing on the issues pertaining to mandarin and its productivity (Tarafder et al. 2017; Gurung et al. 2017), assessing its morphological diversity (Gurung et al. 2020) few efforts have been made to study the wild *Citrus* in Darjeeling and Sikkim Himalayas.

2.1 Systematic significance of pollen morphology of Citrus

Since the year 1753 when Carolus Linneaus established the genus *Citrus*, the taxonomy of *Citrus* and its closely related genera have been highly disputed (Lin sun et al. 2015). At present, there are two principal systems of *Citrus* taxonomy – Swingle and Reece (1967) system and Tanaka (1977). Although Tanaka (1977) believed *Citrus* hybrids, cultivars, bud sports as botanical species, Swingle and Reece (1967) disagreed to consider them as “good taxonomic species”. Ever since the taxonomical ambiguity has been explored by several studies following various techniques (See Section 1.3). Out of the various techniques implemented, studying pollen morphology is important in plant taxonomy (Taia 2020). The use of palynological studies to solve taxonomic problems is gaining traction, and there is a lot of research going on in this field (Inyama et al. 2015; AL-Anbari et al. 2015; Mostafa et al. 2017; Taia et al. 2020). The pollen characters have the probability of bearing high significance at the species and generic levels of taxa or among higher levels (Davis and Heywood 1973; Stuessy 2009). Palynological traits have been used to resurrect several contested genera and to solve questions relating to the origin and evolution of several taxa (Nair 1980). Diversified morphological details of pollen characters like pollen shape, symmetry, size, polar equatorial outlines, number of aperture wall or exine thickness, pore diameter, and exine or wall ornamentation are significant attributes of potential taxonomic importance that are diagnostic at the species level and provide a useful data for the intergeneric classification of the larger genera (Edeoga et al. 1996).

2.2 Significance of floral morphology, biology of Citrus

Heterozygosity is abundant in *Citrus* form that makes breeding *Citrus* very complicated. Such a scenario often leads to taxonomic complications often leading to controversies (Barret and Rhodes 1976). The classifications of *Citrus* by early taxonomists were based on traditional morphology, anatomy, and geographic distribution of taxa. Swingle used fourteen characters while Tanaka used seven characters used by Swingle along with fourteen other characters for the classification (Barret and Rhodes 1976). Later, Singh and Nath (1969) listed 17 characters that they believed to be important for species discrimination. However, there are only four characters (petiolar wing, coherence of stamens, colour of cotyledons, and colour of pulp) that were commonly used which indicated the difference in opinion concerning which characters are important in species discrimination (Barret and Rhodes 1976). However, phylogenetic analysis (Scora 1975; Barret and Rhodes 1976), organeller genome analysis (Green et al. 1986; Yamamoto et al. 1993), and microsatellites (Fang and Roose 1997) has caused a paradigm shift in the way *Citrus* taxonomy is pursued.

Regardless, morphological features were employed to investigate Himalayan *Citrus* diversity (Sharma et al. 2004). The variation between kinnow mandarin and rough lemon was also studied using morphological analysis (Jaskani et al. 2006; Altaf and Khan 2008). Floral biology can help researchers better understand the mechanisms of self-incompatibility and pollen sterility, both of which are crucial in fruit breeding and yield (Kumatkar et al. 2016). Further, morphological assessment to assess diversity has been successfully adapted (Dorji and Yapwattanaphun 2011b; Gurung et al. 2020) in the backdrop that agronomic traits of *Citrus* are controlled by multiple genes that can be assessed only through morphological assessment (Liu and Deng 2007). As a result, while morphological examination may not be sufficient for classifying *Citrus*, it is extremely effective in determining diversity and genetic links across species and cultivars.

3.1 Pollinators and Pollination

Pollinators are insects or vectors that transfer the pollen from male part of flower i.e., anther to the female part of flower enabling fertilization and seed production (Potts et al. 2016; Das et al. 2018). Pollination is an ecosystem service that enhances crop production and helps in sustaining human life (Potts et al. 2016; Klein et al. 2007). Furthermore, many wild plants that provide calories and micronutrients to human diets require pollination as well (Sundriyal and Sundriyal 2004). Approximately 90% of wild flowering plant species worldwide rely on insect vectors for pollen transmission. These plants are essential for ecosystems to function properly because they supply food, habitat, and other resources to a variety of different species (IPBES 2016). One-third of the agricultural plants including the cultivars are dependent on animal pollinators for their reproduction and increased fruit set (Greenleaf and Kremen 2006). Unfortunately, agricultural intensification has led to a loss of habitat of many insect pollinators and monoculture plantings have threatened insect biodiversity (Krebs et al. 1999; Tscharntke et al. 2005). A palpable concern for the global decline in pollinators can be sensed (Biesmeijer et al. 2006) which may affect fruit set or seed set (Rathcke 1983) influencing its yield (Rodrigo and Herrero 1996; Partap et al. 2000). The decline in pollinators would also result in a parallel decline in its associated plant species (Biesmeijer et al. 2006). Insect pollination is therefore also known as an “endangered ecosystem service” (Corbet 1991; Williams 1994; Kevan and Philips 2001; Slujis van der and Vaage 2016). The pollinator crisis threatens global and local food security and can destabilize human life (Slujis van der and Vaage 2016). Therefore, studies in crop pollination are upfront because of the perceived danger that it invites (Ghazoul 2005; Slujis van der and Vaage 2016; Steffan-Dewenter et al. 2005). However, a detailed preview of pollination studies is incomplete or redundant (Klein et al. 2007). Therefore, understanding the pollination needs of different cultivated plants is a prerequisite to understanding their dependency on animal pollinators (Klein et al. 2007).

In this regard, the pollination requirement of *Citrus* has been arising much curiosity. Domestication of *Citrus* spp. along with grafting has resulted in variation in the existing species in comparison to its wild progenitors (Casas et al. 1999; 2007). As such, the present cultivars exhibit tremendous variation in pollination requirements. *Citrus* was considered a crop with little or no requirement for insect pollination (McGregor 1976). However, Webber and Batchelor (1943) emphasized that no variety is ever static, and the possibility that its pollination requirements have changed cannot be ruled out.

However, it has been found that pollination requirements of *Citrus* have been confusing and misleading. It has been reported that *Citrus* have complete or perfect flowers and generally pollinate themselves to produce fruit (Halder et al. 2019). *Citrus* has been reported to exhibit varying breeding systems within species and cultivars of the same species (McGregor 1976; Roubik 1995). Pollinator reliance and breeding systems differ between and within *Citrus* species, and even between cultivars of the same species (Roubik 1995).

Several *Citrus* hybrid cultivars also exhibit sexual incompatibility which affects its yield (Futch and Jackson 2003). Further, *Citrus* exhibit parthenocarpy and is an important trait that results in a seedless variety (Iwamasa and Oba 1980; Li 1980). Therefore, it wouldn’t be wrong to say that the pollination requirements for *Citrus* are quite diverse (Sanford 1992; McGregor 1976). The reason for such a varying breeding system can be attributed to its numerous varieties and the fact that more are being developed all the time. Moreover, each variety has its characteristics that determine the pollination mechanism in them (Sanford 1992). For instance, some studies state that cross-pollination is not necessary for fruit set (Roubik 1995; Krezdorn 1986), whereas others showed that insect pollinators increased fruit production (Burger 1985). Most seeds are produced by asexual means in some cultivars. However, the benefits drawn from insect pollination cannot be negated (McGregor 1976).

3.2 Inflorescence of Citrus and its sexual process

A *Citrus* flower usually comprises both male and female sex organs in the same flower (Futch and Jackson 2003). Each flower has radial symmetry and consists of 5-6 sepals and 4 petals (Fig. 3B). The inflorescence is a cyme or raceme, rarely of solitary flowers. The flowers are usually bisexual and actinomorphic, hypogynous, rarely epigynous. It consists of 4-5 (2-3) sepals or lobes. The corolla is apopetalous or sympetalous with 4-5 (0, 2-3) imbricate or valvate petals or lobes. The stamens are 8-10- ∞ usually diplostemonous in 2(1-4) whorls.

The stigma and ovary are connected by a white style that forms a knob (Cholis et al. 2020). The pistil is in the centre of each flower and is surrounded by a whorl of stamens which consists of long filaments with anthers at the top (Fig. 3A). The anthers are longitudinal in dehiscence and consist of yellow pollen that gets shed on the sticky surface of the stigma (Futch and Jackson 2003). *Citrus* pollen is heavy and sticky (Futch and Jackson 2003), and is not adapted for wind pollination (Free 1970). *Citrus* flowers are either self-pollinated, cross-pollinated (Free 1970), or can produce fruit by parthenocarpy or nucellar embryony (Zhang et al. 2018). At the bottom of the ovary, there is a nectary gland. *Citrus* produces nectar copiously (McGregor 1976) and is secreted continuously for at least 48 hours after the opening of the flower (Hearn et al. 1942).

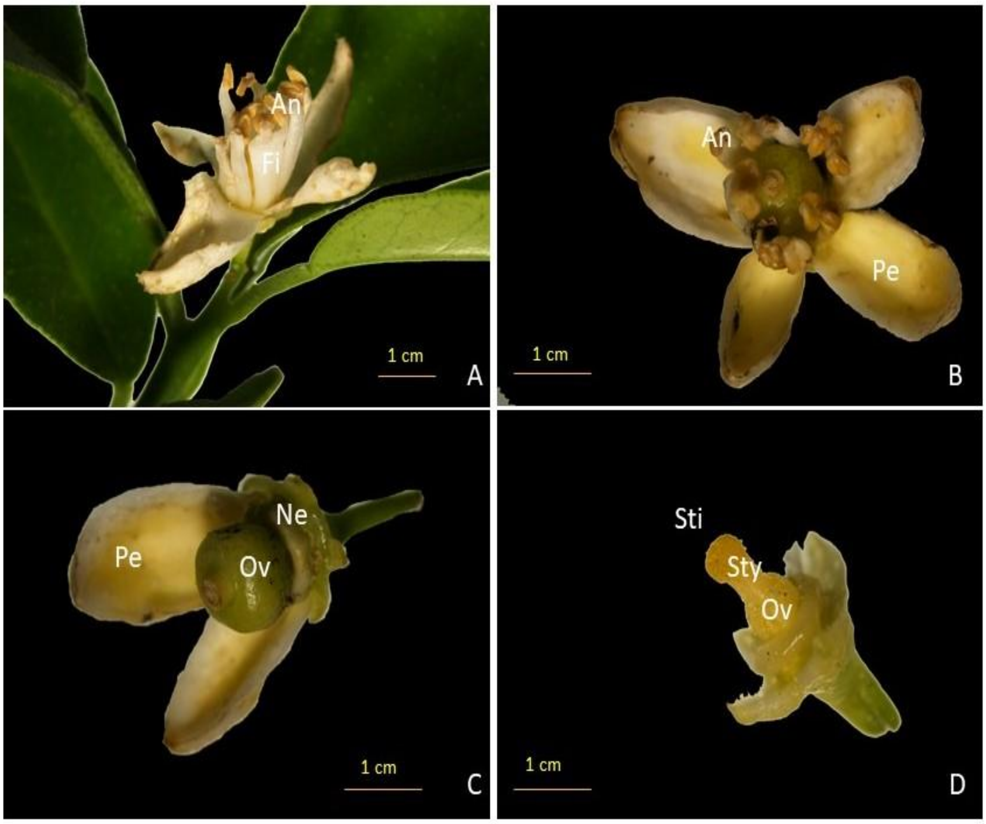


Fig. 3. Flowers of mandarin (*C. reticulata*): **A-B.** Single flower, **C.** fertilized Ovary, **D.** female sex organ (pistil); An- Anther, Fi- Filament, Pe- Petals, Ov- Ovary, Ne-Nectar, Sti- Stigma, Sty- Style. © Subhankar Gurung

3.3 Breeding system of sweet orange

Breeding system of oranges is complex because of the variation in its cultivars. *Citrus sinensis* var. Pera-Rio benefitted from the visitation of bees resulting in heavier, less acidic fruit with fewer seed set (Malerbo-Souza et al. 2003). Further, the fruit production was 35.30% greater in uncovered flowers, fruit weight was higher (180.2g) than covered ones (168.5g) (Malerbo-Souza et al. 2004). However, bees did not affect the production of *C. sinensis* var. Valencia sweet oranges (Francke et al. 1969; Sanford 1992). Sanford (1992) also mentions that Valencia does not benefit much from pollination by bees. Valencia experienced lower pollen grain germination resulting in a lower seed set (Mesejo et al. 2007). Defective ovule mutants encourage flawed pollen tube guidance (Mesejo et al. 2007), and therefore attributed low seed set in Valencia sweet orange to defects during female gametogenesis (Koltunow et al. 1995). However, unlike the previous report, fertilization remains an important factor in its improved fruit set (Mesejo et al. 2007). Similarly, ambiguity regarding the pollination of Washington navel remained when studies demonstrated that cross-pollination might influence fruit set (Krezdorn 1965). However, later studies reinstated that cross-pollination does not affect the yield of Washington navels (Krezdorn 1965). Atkins (1963) stated that there is a possibility that cross-pollination by bees may cause them to retain more fruit (Atkins 1963). There was a 31% increase in the fruit set, 21% increase in fruit weight, 33% more juice, and 36% more seeds from fruits on trees visited by bees (Wafa and Ibrahim 1960). Further Washington Navel is male sterile and is incapable of sexual reproduction when used as a pollen parent (Davenport 1986). Therefore, the likeliness of it requiring a pollinizer is high for a successful fruit set. In this context, it was observed that Washington Navel produced the most fruits when crossed with ‘Grusinian’ orange (Zavrashvili 1967b). However, the embryo sac of ‘Washington Navel’ may also degenerate before pollen tubes penetrate through it.

3.4 Breeding system of Pummelo

Most of the pummelo varieties are self-incompatible because pollen tubes cannot grow properly because of the genetic barrier to inbreeding (Busch and Schoen 2008). Further, most pummelo trees are self-incompatible and should be inter-planted with other cultivars (Aala 1953). Shaddock (*C. maxima* L.) or (*C. grandis* L.) increased its fruit set %, fruit retention, fruit weight, fruit size when cross-pollinated with Balady orange pollen grains (Atawia et al. 2016). Further, Aala (1953) suggested that bees are important for proper pollination and fruit set irrespective of a cultivar being self-fertile or self-sterile. *C. maxima* (burm) Merr cv. nambangan is also reported to be self-incompatible and is dependent on insect pollinators for fruit set (Cholis et al. 2020). However, Hoang et al (2014) reported that pollen tube growth of *C. grandis* cv. Phuc Trach, Dien and Red Pummelo were arrested in the style inhibiting fertilization. Da Xanh and Nam Roi pummelos did not require pollination and produced large-sized seedless fruit by vegetative parthenocarpy (Hoang et al. 2014). Therefore, it is an established fact that few cultivars of pummelo are highly parthenocarpic (Iwamasa and Oba 1980) and pollination is unnecessary for commercial fruit production.

3.5 Breeding system of grapefruit

The pollination requirements and breeding system of grapefruit are poorly understood (Chacoff and Aizen 2007). Some studies suggest that cross-pollination is not a prerequisite for fruit set (Roubik 1995; Krezdorn 1972), whereas others demonstrate that the transfer of cross-pollen mediated by insects increases fruit production (Burger 1985). For example, there are species such as Red Blush grapefruit cultivar that is highly parthenocarpic while Marsh grapefruit set four times as many fruits in open-pollinated flowers than selfed flowers (Wright 1937). Star Ruby grapefruit (*C. paradisi* Macf.) consists of non-functional pollen, non-functional ovules and produces more fruits when cross-pollinated (Burger 1985). Further, studies suggest that pollen transfer by insects and pollen tube development are important factors for fruit production in grapefruit (Crane and Walker 1984; Chacoff and Aizen 2007). Chacoff and Aizen (2007) also demonstrated that more cross-pollen tubes reached the ovary than self-pollen, indicating the importance of the pollinators in successful fruit development.

3.6 Breeding system of Lemons and Limes

Lemons *C. aurantiifolia* (Christm.) Swingle is self-compatible and shows both autogamous and allogamous types of pollination (Karmakar 2013). On the contrary, Tahiti lime is strongly parthenocarpic and its dependency on pollination is feeble for increased fruit set (Krezdorn 1970). Parthenocarpy in Tahiti lime is followed by chromosomal irregularity during meiosis, reflecting the triploid origin of the limes (Soost and Cameron 1980). Likewise, parthenocarpy is high on thornless Rodan lime resulting in a fewer number of seeds (Abrahimy et al. 2004). However, fruit set of *Citrus limettoides* was better in open-pollinated flowers than emasculated or hand-pollinated flowers (Motial 1964). Further, the flower-fruit ratio and fruit-seed ratio of lemons declined in the absence of pollinators than in open-pollinated conditions (Karmakar 2013). The yield is further enhanced in lemon with the planting of a suitable pollinizer. For instance, the yield of Kagzi Kalan'= lemon is supposedly enhanced with the plantation of 10% pummelo along with it (Haldhar et al. 2018).

Studies have reported that *Citrus limon* L. was self-incompatible and fruit and seed set were greater in cross-pollination than self-pollination (Hossain and Rabbani 2011). Further, it has been observed that pollination by bees is not required for the production of fruits (Webber 1930). Further, lemons were reported to be self-compatible that produced fruit by self-pollination without the influence of pollinators (McGregor 1976). Emasculated unpollinated flowers of *C. limon* produced seedless fruits, indicating its parthenocarpic ability (Zhang et al. 2012). Although cross-pollination helped *C. limon* produce fruits with germinable seeds, it was self-pollination that produced seedless fruits (Zhang et al. 2012). However, studies also demonstrated that lemon trees caged without bees produced 42.5% less than open-pollinated trees while trees caged with bees produced 10% less fruit indicating that bees contribute to geitonogamous self-pollination (Zavrashvili 1964).

3.7 Breeding system of Citrons

Citron is reported to be receptive to the pollen of the same species (Moore 2001). Citrons are presumably self-compatible and hence citrons as maternal parents are not common (Moore 2001; Scora 1975). Citrons have the lowest heterozygosity which results in its high rate of selfing (Barkley et al. 2006). It has been observed that the male and female organs of Corsican citrons mature simultaneously before opening of the petals which indicates its self-fertilization mechanism (Luro et al. 2012). As a result, citron propagation by seed in Israeli home gardens seldom results in hybrid seedlings (Goldschmidt 2009). Further, it has also been reported that no pollination problems have been observed on citron (McGregor 1976).

3.8 Status of pollination study and its relevance in Citrus

Citrus is an important cash crop and has a varying degree of pollination requirements. Although, several studies have been conducted to ascertain the pollination requirement of *Citrus* we found uncertainty in the importance of pollination in *Citrus* as far as market demands are concerned. There are several varieties of *Citrus* (Webber and Batchelor 1943) that are poorly known from the pollinators and pollination requirement (Chacoff and Aizen 2007). While McGregor (1976) stated that few studies have been conducted in other groups of *Citrus*, we are in a much better position now than we have been in the past to understand the pollination requirements of the plants. Nonetheless, considering that new cultivars are developed every time, the study will always be in its infancy requiring constant monitoring to understand their breeding system.

4.1 Nutritional importance of Citrus: From Pollination Perspective

Pollinators play an important role in ecosystem functioning and global food security (Slujis van der and Vaage 2016). Furthermore, crop species pollinated by animals supply essential nutrients for human health (Eilers et al. 2011). *Citrus* fruits have a high Vitamin C (ascorbic acid) content (Liu et al. 2012). Other macronutrients found in the fruit include fibre, potassium, folate, calcium, thiamine, niacin, vitamin B6, phosphorus, magnesium, copper, riboflavin, and pantothenic acid (Economos and Clay 1999). *Citrus* fruits comprise several secondary metabolites such as flavonoids, alkaloids, carotenoids, phenol acids, and essential oils (Lv et al. 2015). When compared to other secondary metabolites, flavonoids have proven to be more active (Lv et al. 2015). Furthermore, *Citrus* fruits have anti-oxidant, anti-allergy, anti-cancer, and anti-microbial properties as well. Although the advantage of animals for crop yield is well established (Klein et al. 2007), the impact of animal pollination on the nutritional composition has just been garnering attention (Klatt et al. 2014; Brittain et al. 2014). In their studies, Brittain et al (2014) exhibited the positive influence of pollination on the nutritional and commercial value of almonds. In the study, the authors demonstrated that oleic to linoleic ratios in almonds were higher in cross-pollinated and lowest in pollinator excluded trees. Besides, pollination has also been attributed to improving the commercial-grade, shelf life of fruits (Klatt et al. 2014). When compared to naturally pollinated apples, hand pollination led to greater dry matter content (DMC) levels and a propensity for a poorer (Potassium) K: (Calcium) Ca ratio (Samnegárd et al. 2019).

4.2 Flavonoids

Cinnamic acid derivatives, coumarins, and flavonoids (flavonones, flavones, and flavonols) are the main phenolic chemicals found in *Citrus* fruits (Horowitz and Gentili

1977; Manthey and Grohmann 2001). Flavonoids are reported to have 10,000 structural variants and are biologically active low molecular weight secondary metabolites produced by plants (Weston and Mathesius 2013). Flavonoids can interact with a variety of targets in subcellular sites to trigger varied behaviours in microorganisms, plants, and animals due to their physical and biochemical properties (Taylor and Grotewold 2005; Buer and Djordjevic 2010). Plant-produced flavonoids are significant dietary components in animal systems, and they have been shown to have anti-bacterial, anti-inflammatory, anti-fungal, anti-viral, anti-mutagenic, and anti-cancer effects (Taylor and Grotewold 2005). Flavonoids have also been reported to be effective against Alzheimer’s disease and atherosclerosis (Ovando et al. 2009; Lee et al. 2009).

4.3 Proteins

The proteins are mostly enzymes, which include transferases, hydrolases, lyases, ligases, and oxyreductases in various regions of the fruits, despite their low concentration (Vandercook 1977). The majority of *Citrus* amino acids are considered non-essential based on past research (Block and Bolling 1994). Non-essential amino acids like alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, serine, and proline are found in a variety of orange, lemon, and mandarin varieties, whereas essential amino acids like valine, phenylalanine, threonine, leucine, methionine, and lysine are found in a few orange and grapefruit varieties**.** (Zamorani et al. 1973; Giacomo et al. 1974; Zamorani and Russo 1974; Benk 1975; Russo et al. 1975; Wallrauch 1980; USDA National Nutrient Database 2011). Arginine is the only semi-essential amino acid (Imura and Okada 1998) that exists in moderately measurable concentrations among the several key amino acids found in *Citrus* juices (Attaway et al. 1972). Overall, *Citrus* fruits' free amino acids are unlikely to have a significant impact on human nutrition (Liu et al. 2012).

4.4 Ascorbic acid

Vitamin C is needed for collagen synthesis, the protein that serves so many connective functions in the body. Vitamin C helps in the synthesis of protein, aids the liver in the detoxification of toxic substances in the system, and the blood in fighting infections. Ascorbic acid aids immune protection by supporting both the innate and adaptive immune systems' cellular functioning (Carr and Maggini 2017). As an antioxidant, it reacts with compounds like histamines and peroxides to reduce inflammatory symptoms (Barrita and Sánchez 2013). Its antioxidant properties have been linked to a lower risk of cancer (Lupulescu 1990; 1993). Vitamin C is an effective reducing agent and scavenges reactive oxygen and nitrogen species in the body (Schlueter et al. 2011).

4.5 Antioxidant Capacity and Radical Scavenging Effect

The fundamental cause of numerous illnesses in humans is free radicals and reactive oxygen species (ROS), which are produced when there is an imbalance between the synthesis and neutralization of prooxidants, resulting in oxidative stress. (Gangwar et al. 2014). They damage lipids, proteins, and DNA, resulting in a variety of chronic disorders in humans, including cancer, diabetes, ageing, and other degenerative diseases. (Harman 1998; Maxwell 1995). Human cells produce enzymes like superoxide dismutase (SOD) and catalase, as well as substances like ascorbic acid, tocopherol, and glutathione, to defend themselves from free radical damage. (Nikl et al. 1994). Much attention has been directed towards plants for the development of ethnomedicines as they contain phenols, flavonoids, alkaloids, tannins, vitamins, terpenoids, and many more phytochemicals responsible for different pharmacological activities (Rice-Evans et al. 1995). According to a recent study, natural antioxidants have been linked to a lower risk of cancer and a variety of other ailments (Gerber et al. 2002).

Flavanones, in particular naringin, which gives grapefruit its bitter flavour, is one of the most frequent flavonoids found in *Citrus* fruits (Ross and Kasum 2002). Before absorption, gut bacteria hydrolyze naringin to naringenin, which is a common precursor to numerous flavonoid classes (Shulman et al. 2011). Naringenin is expected to provide a variety of biological and physiological benefits, including serving as an antioxidant (Shulman et al. 2011).

With this background, the proposed work will dwell on morphological characterization of mandarin grown in Sikkim and Darjeeling Himalayas and understanding its adaptation in its requirement of pollination for a better yield.

The major objectives of the work are:

1. **Morphological identification and diversity of mandarin (***Citrus reticulata* **Blanco) in Darjeeling and Sikkim Himalayas.**
2. **Assessment of floral biology of mandarin.**
3. **Assessment of pollination biology of mandarin.**

According to farmers' anecdote, mandarin cultivation is in practice for decades, with trees as old as 80 years. Unfortunately, there is a paucity of knowledge and data about the cultivars that their forefathers planted in the field, resulting in ignorance about mandarin cultivars. The majority of the new plantations are from seeds of diverse and unknown origin. In the Himalayas of Sikkim and Darjeeling, there is no real history of *Citrus* farming. In **Chapter 4**, I examine the morphological diversity of *C. reticulata* grown in Sikkim and Darjeeling Himalayas. Fruits, leaves, and flowers were collected and qualitative and quantitative data were pooled to understand the similarity-dissimilarity of the accessions. I build relationship of them with the help of cluster analysis, grid mad and identify regions with superior germplasms and identify the important variable contributing to variation. **Chapter 5** is an add-on of Chapter 3, wherein we try to assess and categorize mandarin on the basis of floral characteristics. Further, I dive deeper into pollen characteristics of mandarin which is a significant diagnostic attribute at the species level and provides useful data for the intergeneric classification of the larger genera. In addition, I delve deeper into mandarin pollen features, which are considered to be an important diagnostic attribute at the species level. In **Chapter 6**, first I try to identify the most important pollinator of Sikkim mandarin and Darjeeling mandarin and try to understand the pattern of its visit to the flowers during the day. Alongside, I try to understand their adaptation through ages of documentation and understand its pollination requirements for successful reproduction.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Cultivation of Mandarin in India

In India, mandarin orange *Citrus reticulata* Blanco contributes about 36% of the country's total production of *Citrus* fruits (Singh 2001), covering an area of 428 thousand hectare (ha) with an annual production of 5101 thousand tons (Yadav and Jain 2019). The North-Eastern Hill Regions (NEH), which consists of eight Indian states and covers 1600 ha of mostly mountainous terrain, is one of the country's three primary centres of mandarin production and makes a substantial contribution to the small farm economy (Singh 2001). The Indo-China landmass, which includes the NEH region of India bordering south China, is regarded as one of the world's genetic diversity hotspots (Ghosh and Singh 1993). Some high-quality unique ecotypes of *C. reticulata*, usually known as Darjeeling mandarin, Sikkim mandarin and Khasi mandarin, are traditionally farmed in this area and are the subject of crop improvement programmes utilising conventional methods (Ghosh and Singh 1993; Singh and Huchche 2001).

2.2 Morphological analysis of Citrus

There are several methods used for the assessment of diversity and genetic relationships among the accessions. Several molecular marker-based techniques which are stable and independent of environmental conditions are used cultivar identification (Novelli et al. 2000), phylogenetic analysis (Pang et al. 2003; Dorji and Yapwattanaphun 2015), genetic polymorphism (Golein et al. 2005; Das et al. 2004b) and zygote and nucellar identification (Oliveira et al. 2002). Despite its reliability, morphological study is still considered important and is used for cultivar identification and diversity analysis (RodrÌguez-Garay et al. 2009; Elameen et al. 2011). Further, Susandarini et al (2013) highlighted the importance of morphological studies in horticultural plants for cultivars identification. Several studies combining morphological and molecular markers have shown to be complementary for mandarin characterization (Campos et al. 2005). Further, most of the *Citrus'* physiological and morphological traits are governed by "Quantitative trait locus" (QTLs) which can be accessed only through morphological assessment (Liu and Deng 2007; Dorji and Yapwattanaphun 2011a). Morphological markers have been deployed for estimation of diversity and cultivar identification of watermelon (Huh et al. 2008), sweet potato (Elameen et al. 2011), agave (RodrÌguez-Garay et al. 2009), physic nut (*Jatropha curcasi*) (Shabanimofrad et al. 2011). Knowledge of genetic linkages, as well as proper genotype selection and identification, is essential for evolutionary studies, germplasm collection, and crop improvement programmes. (Choudhary et al. 2014).

In *Citrus*, morphological analysis is used to identify superior rootstocks that can resist climate change, diseases (Jaskani et al. 2006), study variation between kinnow mandarin and rough lemon (Altaf and Khan 2008). Further, morphological characters have also been used for the numerical taxonomic study of *Citrus* and *Fortunella* Swingle (Handa and Oogaki 1985). In addition to the importance of morphological markers in agronomic traits, the technique is widely used due to convenience and cost-effectiveness.

2.3 Genetic diversity of Citrus mandarin: Understanding the dynamics

Mandarin (*C. reticulata*), grapefruit (*C. grandis*), and citrons *(C. medica*) are considered the three true species (Campos et al. 2005). Among the three true *Citrus* mandarin groups are the most phenotypically heterogeneous and consist of several species along with intergeneric and interspecific species (Moore 2001). Mandarin is the second most important *Citrus* crop globally (Pal et al. 2013). One of the reasons attributed to wide variation could be the occurrence of high percentage of zygotic twins (Das et al. 2007). Such phenomenon has resulted not only in wide phenotypical variation but also lack of uniformity in fruit quality (Dorji and Yapwattanaphun 2011a). Such variation can also be attributed to a high frequency of bud mutations and aeons of domestication and wide dispersion (Scora 1988).

The study of genetic variability in *Citrus* is pertinent for characterizing germplasm, preventing genetic erosion, and identifying new cultivars (Barkley et al. 2006). Morphological markers were used for cultivar identification (Dorji and Yapwattanaphun 2011a) and morphological diversity (Dorji and Yapwattanaphun 2011b) of local mandarin of Bhutan. The morphological analysis indicated wide variation in mandarin accessions and concluded that mandarin in Bhutan might be a variation from a single clone. On the contrary, amplified fragment length polymorphism (AFLP) analysis concluded that the probability of Bhutanese mandarin comprising of clones of a single variety is low (Dorji and Yapwattanaphun 2015). Random amplified polymorphic DNA (RAPD) profiling of mandarin of Northeastern Himalayas by Das et al (2004b) showed a wide genetic diversity of mandarin orange in Northeast India that can be used in plant improvement programmes. Similarly, high genetic diversity was observed using inter simple sequence repeats (ISSR) primers among the cultivated

Khasi mandarin grown in Northeast India suggesting orchards to be an effective reservoir of genetic germplasm (Kashyap et al. 2021). Leaf length, stamen length, fruit weight, equatorial diameter, polar diameter, and juice percent significantly contributed to the genetic divergence of 50 Naga mandarin genotypes (Longkumer and Kabir 2013). Similarly, variations of local mandarin of Bhutan were attributed to variations in the characters of leaves, flowers, and fruits (Dorji and Yapwattanaphun 2011b).

Golein et al (2005) used Simple Sequence Repeats (SSR) markers to conclude Ponkan and Dancy to be similar cultivars of *C. reticulata* in contrast to Tanaka's (1954) suggestion of them being separate species. Pal et al (2013) concluded that morphological and molecular markers can be used with success to determine genetic variability and relationship in mandarin. Studies conducted by Kijas et al (1997), Novelli et al (1999), Ruiz et al (2000) showed that SSR markers were ineffective to distinguish *C. sinensis* cultivars but were efficient in *C. reticulata* cultivars. These results reflect the breeding history of *C. reticulata* (Golein et al. 2005) and show its heterogeneity with cultivars ranging from facultative apomicts to completely sexual types (Barret and Rhodes 1976).

Study conducted by Altaf et al (2014) showed that genetic diversity of mandarin can also be associated with the seeded and seedless strains where Kinnow mandarin showed variations in fruit shape, growth habit, fruit texture, TSS, and ascorbic acid in seeded and seedless fruits. Further SSR analysis exhibited 81.62% polymorphism in seeded and seedless varieties.

2.4 Taxonomy of mandarin: Current understanding

Mandarins are the most diverse category of *Citrus* fruits, with monoembryonic and polyembryonic cultivars as well as a wide range of interspecific hybrids (Hodgson 1967; Barret and Rhodes 1976). According to Tanaka (1969; 1977), there are 36 mandarin species whereas Swingle recognized only three species, one of them being *C. reticulata* Blanco (Swingle and Reece 1967). However, according to Duran-Villa (1997), there are five commercially important *Citrus* species that are mandarins. Unresolved taxonomic problems have arisen as a result of the mandarins wide variation. For instance, Tachibana has long been classified as a separate species in most classification systems (Swingle and Reece 1967; Tanaka 1954). However, sequence analysis shows its close affinity towards *C. reticulata* (Shimizu et al. 2016; Hirai et al. 1990) and does not identify its taxonomic position as a separate species (Wu et al. 2018). Further, both chloroplast and nuclear genome phylogenies evidently distinguish Tachibana from the mainland Asian mandarins (Wu et al. 2018). This proposes Tachibana to be designated as a subspecies of *C. reticulata* (Wu et al. 2018). Furthermore, pummelo introgression into mandarin genetics separated the mandarins into two groups, one with 1-10% pummelo admixture and the other with 12-38% pummelo genes (Talon et al. 2020). The first group contains traditional mandarins such as Cleopatra, Sunki, Changsa, Dancy, Ponkan while more modern mandarins such as King, Wilking, Fallglo, Kiyomi, Satsuma, Clementine are associated with the second group (Talon et al. 2020). The existing conflicting *Citrus* taxonomy, which is based on long-standing, contradictory recommendations, is in desperate need of a thorough reformulation that takes into account the hybrid/admixture nature of cultivated *Citrus*.

According to Campos et al (2005), correct accessions classification and identification in a germplasm bank allow for the resolution of management issues such as avoiding duplication in the exchange and conservation of germplasm within the bank, detecting mislabelled accessions, certifying propagated material, and inferring the genetic variability represented by the collections to increase or maintain an appropriate range of genetic diversity.

Table 1: List of different *C. reticulata* cultivars

Cultivars	**Country**	**Citation**
Kinnow	Pakistan, United States of America (USA)	Saleem et al. 2005; Pal et al. 2013; Khalid et al. 2019
Feutrell's Early	Pakistan	Saleem et al. 2005; Pal et al. 2013
Wuzishatangju	China	Ye et al. 2009
Shatangju	China	Miao et al. 2013
Batangas	Brasil	Cornèlio et al. 2003
Cravo	Brasil	Cornèlio et al. 2003
Oneco	Brasil	Cornèlio et al. 2003
Ponkan, Dancy	Brasil, China	Cornèlio et al. 2003; Ye et al. 2009; Golein et al. 2005; Pal et al. 2013; Kishore et al. 2012
Chutianju	China	Ye et al. 2009
Sun Chu Cha Kat	India, China, Japan	Wu et al. 2018
Tachibana	Taiwan, Japan	Tanaka (1931); Talon et al. 2020
Darjeeling mandarin	India	Gurung et al. 2020
Mohali	Iran	Golein et al. 2005
Bami	Iran	Golein et al. 2005
Atabaki	Iran	Golein et al. 2005
Mohali I	Iran	Golein et al. 2005
Laddu	India	Pal et al. 2013
Sz-In-com	USA	Pal et al. 2013
Wilking	USA	Pal et al. 2013
Orlando	USA	Pal et al. 2013
Temple	Jamaica	Pal et al. 2013

Fairchild	USA	Pal et al. 2013
Fremont	USA	Pal et al. 2013
Orange East Indian	India	Pal et al. 2013
Nasnaran	Indonesia	Pal et al. 2013
Dweet tangor	USA	Pal et al. 2013
Minneola	USA	Pal et al. 2013
Nagpur mandarin	|India	Pal et al. 2013
Khasi mandarin	India	Pal et al. 2013; Kashyap et al. 2021; Dorji and Yapwattanaphun 2011b
Sikkim mandarin	India	Pal et al. 2013; Dorji and Yapwattanaphun 2011b; Kishore et al. 2010; Kumar et al. 2011
Naga mandarin	India	Longkumer and Kabir 2014
Daisy	USA	Singh et al. 2016b
Nova	USA, Cyprus	Georgiou 2000
W. Murcott, Afourer, Nadorcott	USA, Morocco	Singh et al. 2016a; Gambetta et al. 2013
Limau Madu	Malaysia	Elcy et al. 2012
Sainampueng	Thailand	Stuetz et al. 2010
Shogun	Thailand	Chelong and Sdoodee 2013
Coorg	India	Tripathi et al. 2016
Fortune	Spain	Pèrez-Jimènez and Pèrez-Tornero 2021
Robinson	Turkey	Yildiz et al. 2013

2.5 Application of DIVA-GIS: Mapping diversity

Geographic information system (GIS) mapping is a powerful tool to validate species occurrence (Flemons et al. 2007), preliminary diversity assessment, and recognize gaps in the collection (Pradesh et al. 2010). The DIVA-GIS software analyses gene bank and herbarium records to uncover genetic, ecological, and spatial trends in agricultural and wild species distribution (Hijmans et al. 2001). DIVA-GIS can also be used to check existing coordinates using overlays of the collection-site and administrative boundary databases. DIVA-GIS can be used to overlay coordinates of collection sites over administrative boundary databases for diversity analysis (Hijmans et al. 2001). The mapped points are used with the aid of point-to-grid option to represent the ‘diversity’ and ‘coefficient of variation’ in the form of grids for the traits that are selected (Nizar et al. 2014). DIVA-GIS has been used to identify areas of high diversity in wild potatoes (Hijmans et al. 2000), *Jatropha curcas* (Sunil et al. 2009), piper (Parthasarathy et al. 2006), and soybean (Gai et al. 2005). In addition, GIS has been used successfully to study the geographic distribution of cultivated crop species as well as pest infestation of agricultural crops (Hijmans and Spooner 2001; Ganeshaiah et al. 2003).

2.6 Floral Biology*: *Historical perspective

Charles Darwin was a pioneer in exploring floral biology since 14% of his publications were based on it (Schneckenburger 2009; Rech and Westerkamp 2014). Further, Kölreuter (1761) and Sprengel (1793) works based on Darwin’s work managed to marry evolutionary theory and floral morphology (Schneckenburger 2009; Rech and Westerkamp 2014). By the end of the 19th century, the annals of research on floral biology were compiled by Knuth (1898-1905) indicating the possibilities of the aforesaid studies to become mainstream research field in the new 20th century (Baker 1983). On the contrary, the study on floral biology fell into relative dormancy for nearly fifty years (Vogel 2007). During this period, progress was made in honing tools such as understanding bee behaviour, bird pollination, flower adaptation, breeding, and plant genetics (Proctor et al. 1996; Vogel 2007). However, since the 1960s, floral biology has experienced a renewed interest that still continues (Vogel 2007). The renewed interest was inclined towards interdisciplinary studies, integrating genetics, plant physiology, and molecular biology to explain floral biology better (Vogel 2007;

Oliveira and Rech 2018). At present, floral biology is considered important to gain insights into plant evolution and diversity as a whole (Friis et al. 2011; Sauquet et al. 2017).

2.7 Floral Biology in mandarin

Morphological study is a pertinent assessment to understand diversity and classification (Dorji and Yapwattanaphun 2011b). Under morphological assessment, floral biology is the science of flowers that incorporates floral traits, anthesis, pollen fertility, dehiscence of anthers, and stigma receptivity (Kalloo 1988). In case of mandarin, assessing floral morphology is an important assignment for a *Citrus* breeder (Singh et al. 2016a). According to Ahmad (1996), garnering knowledge on floral morphology, biology, anthesis, pollen fertility, and stigma receptivity are important pre-requisite of efficient hybridization.

The mandarin flower is perfect where both male and female flowers are in the same flower (Krezdorn 1978) (Fig. 4). However, exceptions are lime, lemon, and citron that have staminate flowers (Purseglove 1968), and satsumas that bear pistillate flowers (Kihara 1951). The female sex organ (pistil) is in the centre of each flower. The style unites the ovary at the base with the globose yellow stigma at the apex (McGregor 1976). The flower comprises usually five gloss, flared petals (McGregor 1976). The nectary that produces nectar is situated above the point of attachment of stamens and below the ovary (McGregor 1976; Cholis et al. 2020). There are twenty to forty upright stamens with yellow anther sacs on the top (McGregor 1976). The flower anthesis was reported from 9:00 h to 16:00 h with the peak period at noon (Randhawa et al. 1961).

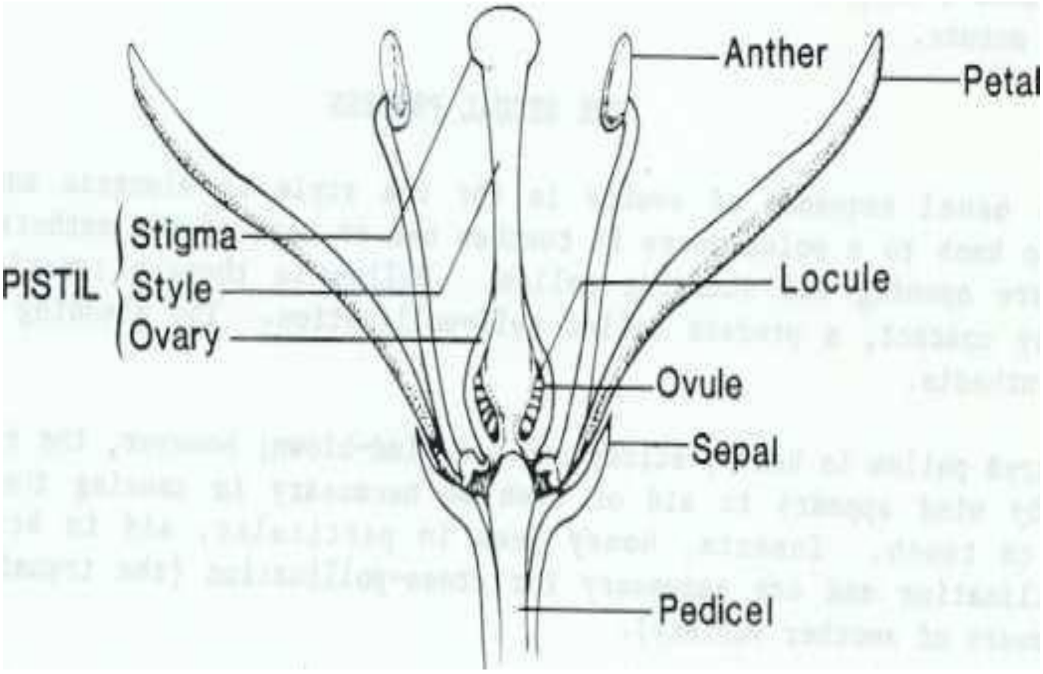


Fig. 4. Diagrammatic representation of open mandarin flower (Source: Krezdorn 1978)

Several studies have been conducted where the morphological attributes of different *Citrus* plants have been characterized to identify genotypes that can be used for breeding or identify new varieties. The attributes associated with plants, flowers, fruits, and leaves have been used by several authors (Domingues et al. 1999) to describe and characterize mandarin varieties and hybrids. Dorji and Yapwattanaphun (2011b) reported no significant variations in the qualitative and quantitative measurements of flowers of *C. reticulata*. Singh et al (2016b) demonstrated the genetic diversity of mandarin accessions with the aid of floral morphology. A significant difference was found in the blooming period, flower diameter, flower length, pedicle length, and calyx diameter. Similar characterization of lemon, grapefruit, and mandarin genotypes have been done by Demir et al (2015), Baswal et al (2016), and Singh et al (2016a) respectively.

The taxonomy and systematics of *Citrus* is complex and most of the named species are clonally propagated hybrids (Dorji and Yawattanaphun 2011b). In such a situation knowledge of floral morphology, biology would be useful in taxonomical studies (Randhawa et al. 1961). Further, palynological study was considered an important tool in solving taxonomic problems. Pollen morphological studies have been adopted by Avci et al (2013) and Inyama et al (2015) and Taia et al (2020) in differentiating members of *Onobrychis* (Fabaceae) and *Citrus* (Rutaceae) respectively. Mary and Gopal (2018) used pollen morphology to study *Ehretia pubescens* and *Cormona retusa* and established it as an important tool to identify between them. Similarly, Taia and Sheha (2001) have used the palynological study to differentiate among *Astragalus* and *Atriplex* species respectively.

2.8 Pollination mechanism of mandarin

The reproductive structures of most flowering plants have faced huge diversification events which have led to adaptions ensuring reproductive success in various environments (González et al. 2018). The changes in the reproductive system result in changes in morphological and floral traits which ultimately lead to alteration in pollinator interaction, affecting their reproductive success (González et al. 2018). Understanding floral biology is important in understanding the dynamics of pollination, reproduction, and breeding (Fayeun et al. 2016).

Angiosperms reproductive system range from sexual to asexual forms (Fayeun et al. 2016) which is determined by the species being hermaphrodite in which male and female reproductive structures are located in the same flower or same plant to dioecious where unisexual flowers are borne on different species (González et al. 2018). The intrinsic properties of plant breeding systems and extrinsic interactions between plants and their pollen vectors govern sexual reproduction (Dafni et al. 2005). Depending on these variables, the plant is either self-pollinated or cross-pollinated.

The cross-pollinated nature of the plant and propagation through seed without nucellar selection, traditionally followed in this region have produced considerable variation. This wide variation in phenotypic characters like plant types and fruit quality parameters is apparent even among the plants within the same orchard (Das et al. 2003). In such a scenario, the pollination requirement of *Citrus* has been ambiguous and a matter to be understood intricately. Among the *Citrus* group, pollination has been reported to be critical in mandarin and mandarin hybrid-complex (Sanford 1992).

2.9 Breeding system of mandarin and mandarin hybrid-complex

Several mandarin hybrids of fine quality have been evolved that have become commercially important varieties. For instance, crosses between clementine and tangelo (*C. reticulata* × [*Citrus paradisi* × *C. reticulata*]*)* released Robinson, Lee, Osceola, Page, and Nova (Reece et al. 1964). King mandarin (*C. nobilis*) proved to be an exceptional parent when crossed with other mandarin types in producing well-flavoured hybrids (Cameron and Frost 1968). Tangelo is a cross between tangerine or mandarin orange (*C. reticulata*) with either grapefruit (*C. paradisi*) or pummelo (*C. grandis*) (Webber & Batchelor 1943). Clementine (*Citrus clementina*) is a hybrid between Mediterranean *Citrus deliciosa* and sweet orange (Atawia et al. 2016). While the hybrids were being released, issues in its pollination were recognized by Lacarelle and Miedzyrzecki (1937) where the fruit set in Clementine mandarin was fewer in flowers enclosed in self-pollination without bees than those flowers enclosed with bees. Clementine mandarin was identified to be self-incompatible but showed improved fruit characteristics when cross-pollinated with March grapefruit and Balady orange (Atawia et al. 2016). Besides, it has also been reported that Clementine mandarin develops a seedless fruit if cross-pollination does not occur (Mesejo et al. 2013). Clementine mandarin cultivars (*C. clementina*) exhibit facultative parthenocarpy and are pollination independent (Mesejo et al. 2013). However, Clemenules exhibits weak parthenocarpic ability than Marisol indicating a difference in the parthenocarpic ability of Clementine cultivars. Further, studies on Lee, Page, Nova and Robinson identified them to be self-incompatible (Hearn et al. 1969). Afourer mandarin was also found to be self-incompatible and produced relatively high fruit set by facultative and autonomous parthenocarpy (Gambetta et al. 2013). On the contrary, Reece and Register (1961) stated that Osceola was not completely self-incompatible. Miwa (1951) demonstrated that Hyuganatsu mandarin was self-sterile but cross-fertile. Several experiments demonstrated that Satsuma mandarin benefitted 6.3% by bees (Zhgenti 1956) and 7-11% as reported by Zavrashvili (1967a; 1967b). They also present obligate parthenocarpy (Vardi et al. 2008), and thus the sexual process is not always required for successful fruit development. Mesejo et al (2007) reported no significant difference in the final fruit set between parthenocarpic and fertilized Satsuma mandarin. Several mandarin cultivars also benefit tremendously from compatible pollinizers for successful fruit development. For instance: grapefruit pollen is an efficient pollinizer in fertilizing Clementine mandarins. Hearn et al (1969) observed that pollens derived from Lee enhanced the fruit size of Page cultivars. Minneola tangelo increased its yield when cross-pollinated with Seminole or Lake pollen which indicated that Minneola fruit set can be enhanced by planting a suitable pollinizer (Mustard 1961).

Some of the accessions of mandarin such as Kunenbo are self-incompatible because of incompatible alleles originating from pummelo (Ngo 2001) while mandarins that originated in China and India are self-compatible (Yamamoto et al. 2006).

2.10 Nutraceutical dependency on pollination

Pollinators are directly responsible for 40% of the supply of micronutrients to the world (Eilers et al. 2011). Smith et al (2015) suggested an increased number of cases of non-communicable diseases and micronutrient deficiency due to the loss of pollinators. This alone suffices to make us realize the importance of pollinators' role in supplying micronutrients. *Citrus* fruits contain vitamins, minerals, fibre, and phytochemicals such as carotenoids, flavonoids, and limonoids (Turner and Burri 2013). Although pollination's role in the nutraceutical composition of *Citrus* fruits has not been conducted, Brittain et al (2014) reported an increase in oleic to linoleic ratio in almonds which is considered most favourable by the customers for health benefits. Pollination also increased the shelf life and commercial value of strawberries (Klatt et al. 2014). There are several investigations of the benefits of animal-mediated pollination on crop yield (Klein et al. 2007; Klatt et al. 2014). However, the benefits of pollination to the nutritional composition of a crop are few (Klatt et al. 2014; Garratt et al. 2013).

CHAPTER 3

STUDY AREA

3.1 Darjeeling and Sikkim Himalayas

The Darjeeling-Sikkim Himalaya marks the beginning of the eastern Himalaya. It is situated between Bhutan in the east and Nepal towards the west. The hilly region of Darjeeling and Kalimpong in West Bengal is located between latitude 27° 13’ N to 26° 27’ N and longitude 88° 53’E to 87° 59’ E and is spread over 3149 km^2 (Roy et al. 2018). Sikkim on the other hand is the second smallest state in India and covers an area of 7096 km^2 (Pradhan 2018). Darjeeling and Kalimpong districts are key geological components of Sikkim, and their physiography and geology are inextricably linked (Basu 2013). Therefore, the state of Sikkim along with the hilly districts of Darjeeling and Kalimpong constitute Darjeeling and Sikkim Himalayas.

3.2 Sikkim

Sikkim as a state lies towards the Northeastern part of India and was merged with India in 1975. Sikkim is very important as it shares international boundaries with Bhutan, China, and Nepal (Sikkim SAPCC 2011). Geologically, the state of Sikkim comprises the rocks of Lesser, Central, and the Tethys Himalaya (Gansser 1964). The altitudinal gradient of the state is the steepest (Schaller 1977), which has resulted in the formation of varied microclimatic conditions resulting in an assemblage of a variety of vegetation and wildlife (Chettri et al. 2001). As a result, Sikkim has been considered as a treasure house of plant diversity in India (Singh and Chauhan 1997) and a ‘biodiversity hotspot’ (Brooks et al. 2006). The vegetation complex of the state is represented by the Sal

(*Shorea robusta*) forest (300-900 m a.s.l.), Chir pine (*Pinus roxburghii*) forest (500-900 m a.s.l.), subtropical forest (300-900 m a.s.l.), warm broad-leaved forest (900 – 1700 m a.s.l.), alder forest (1500-2000 m a.s.l), evergreen oak forest (1700-2800 m a.s.l.), dwarf bamboo thicket (2600-3100 a.s.l), mixed conifer forest (2700-3100 a.s.l.), conifer forest (2800-3700 m a.s.l.) and alpine meadow (4500-5500 m a.s.l.) (Champion and Seth 1968; Fig. 5).

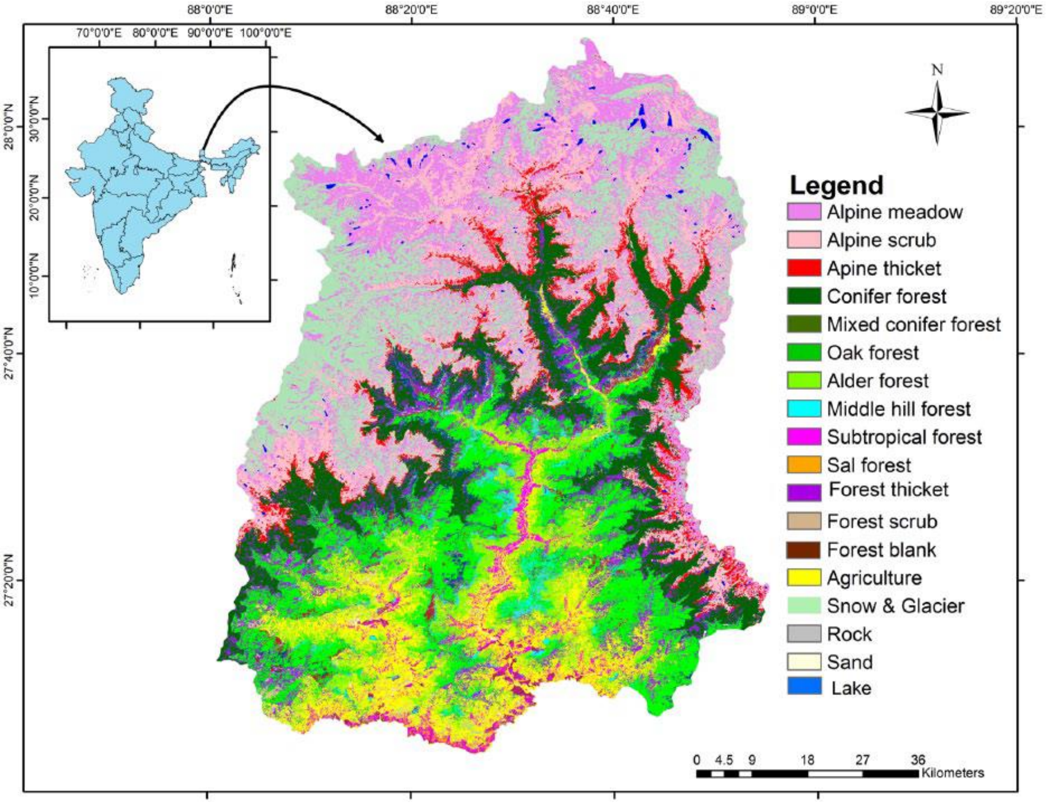


Fig. 5. Land use land cover of Sikkim of north-east India (Source: Dubey et al. 2017)

3.3 Darjeeling and Kalimpong districts of West Bengal

Darjeeling and Kalimpong districts are located in the northernmost part of West Bengal (WB) in the eastern Himalayas, India. The districts are bordered on the west by Nepal, on the north by Sikkim, and on the east by Bhutan (Das et al. 2008). The terrain of the study area comprises both plain and hilly regions. The hill area is formed by recent rock structure and the soil structure is extremely varied due to geolithology, degree of slope, elevation, and vegetative cover (Pramanik 2016). The average mean maximum and minimum temperature of the region ranges from 14.9° C to 8.9° C and the average annual long-term precipitation is 2800 mm (Rather and Bhat 2018).

The soil of Darjeeling district falls under Warm Prehumid Eco-Region under the Eastern Himalayas Agro Ecological Sub Region (DSR 2021a). Soils on the district's highlands, which span from west to east along most of the district's interfluvial areas, are mostly mixed sandy loam and loamy, whereas those on the southern slopes of Mirik and Kurseong are mostly clayey loam and reddish in colour. Sandy soils are mostly found east of the Teesta River (Sarkar and De 2017). In the Kalimpong district, the soil is heterogeneous in nature. It is classed as lithic and typic haplustepts, and its texture varies from gravelly loam to loam. Alfisols are the most common, accounting for 52.96% of the total area, followed by Ultisols (36.27%) (DSR 2021b).

Due to significant climatic, edaphic, and physiographic fluctuations, a diverse range of vegetation structures with highly rich plant and animal diversity has emerged (Das et al. 2008). The present land use and land cover pattern determined vegetation and tea plantation to cover 64.91 % of both the districts (Pramanik 2016; Fig. 6). Further, there are five protected areas in both the districts namely Singhalila National Park, Senchel Wildlife Sanctuary, Mahananda Wildlife Sanctuary, Neora Valley National Park, and Jorepokhari Salamander Sanctuary, and houses an estimated vascular flora of 2912 (Das et al. 2008). The protected area comprises 10% of the total area of Darjeeling and Kalimpong districts (Pradhan 2020).

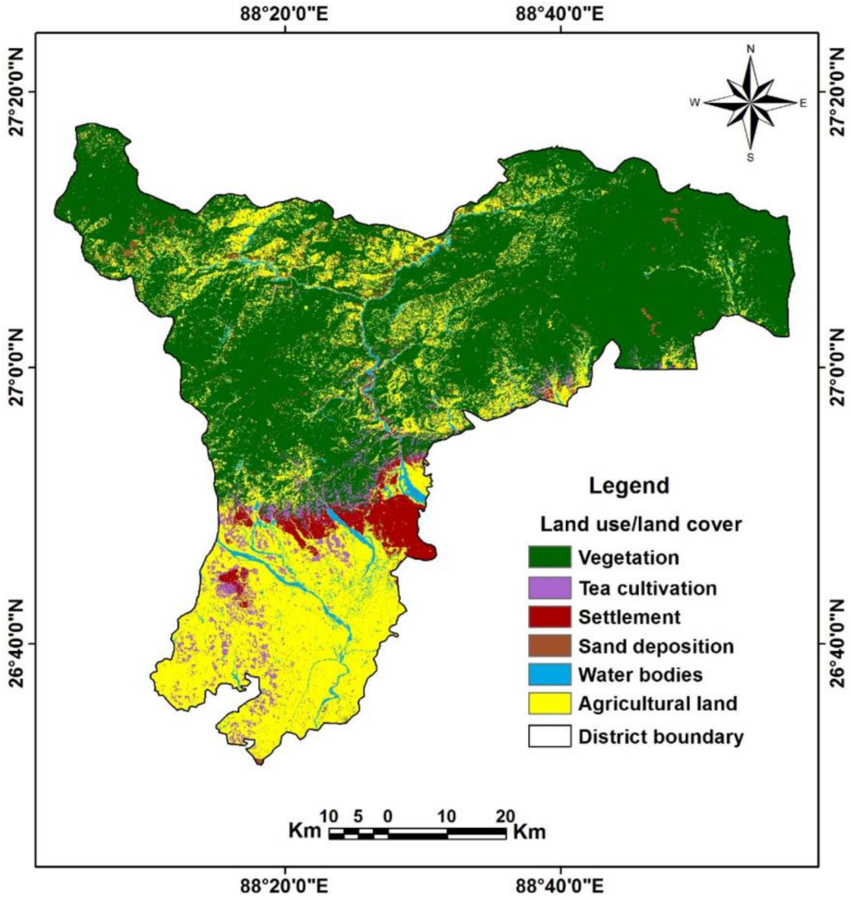


Fig. 6. Land use land cover of Darjeeling and Kalimpong District, West Bengal (Source: Pramanik 2016)

3.4 Agricultural practices

It is in the region of Sikkim and Darjeeling Himalayas that mandarins have been under cultivation since time immemorial. Approximately 67 % of the workforce in Sikkim is employed in the agriculture sector (Joshi 2004). Sikkim is the first Indian state to adapt organic farming, prohibiting farmers from using any chemical inputs in their operations. Wheat, millet, rice, mustard, soybeans, and various vegetables are some of the key agricultural products (Pradhan 2018). However, the important cash crops include large cardamom, mandarin, and ginger (Rahman and Karuppaiyan 2011).

While tourism and the tea industry are major contributors to Darjeeling's economy (Datta 2010), agroforestry is an important component of the farming system (DSR 2021a). Cultivators account for 7.6% of the overall working population in Darjeeling, 4.7 % for agricultural labourers, and 29.96 % for associated agricultural activities. Orange, ginger, pineapple, cardamom, potato, and even vegetables are the district's main revenue crops. More than 80 % of Kalimpong's residents rely on agriculture for a living. Paddy, maize, millet, pulse, oilseed, and potato are among the major agricultural products. Nevertheless, Kalimpong is better recognised as a centre for cash crops like ginger, cardamom, betel nut, and oranges (DSR 2021b).

3.4.1 Sikkim mandarin and its cultivation

Sikkim mandarin belongs to a delicious variety of mandarin groups native to Sikkim (Pradhan 2018). The major *Citrus* growing belts are southern part of north district, central part of south Sikkim, central part of east Sikkim, and southern part of west Sikkim (Kishore et al. 2010). Mandarin is mainly grown in the lower hills of Sikkim under sub-tropical humid climatic conditions and is the most important cash crop of Sikkim (Kishore et al. 2010; Pradhan and Devy 2018). After the decline of large cardamom yield in the last decade (Sinu and Shivanna 2007a; Partap et al. 2014) the Government of Sikkim's Department of Horticulture and Cash Crops has expanded the area under mandarin cultivation (Pradhan 2018). Therefore, the area of mandarin farming in Sikkim increased from 4000 hectares (ha) in 2001-2002 to 12000 ha in 2015-2016 (Fig. 7).

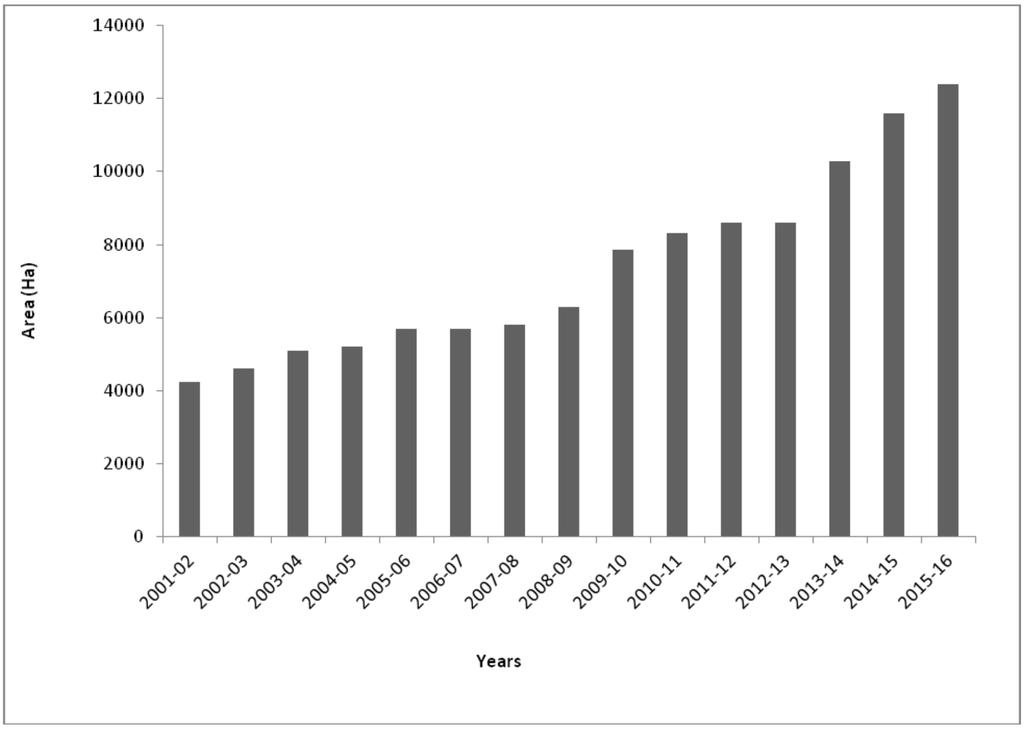


Fig. 7. Area (ha) under Sikkim mandarin orange cultivation (Source: The Department of Horticulture and Cash Crops, Government of Sikkim; Pradhan 2018)

3.4.2 Darjeeling mandarin and its cultivation

In West Bengal, mandarin occupies an area of 4.14 thousand ha with a production of 40.18 thousand tons and productivity of 9.71 thousand tons per ha (Horticultural statistics 2018). In Darjeeling and Kalimpong hills of West Bengal, mandarins have been under cultivation since time immemorial. However, the area and production of mandarin have been decreasing over the years. In 1999-2000, the mandarin cultivated area was 30000 ha. In 2007-2008, it was 1972 ha and now 1600 ha area is under Darjeeling mandarin (Tarafder et al. 2017).

CHAPTER 4

Morphological diversity of *Citrus reticulata* Blanco (mandarin) in Darjeeling and Sikkim Himalayas

4.1 INTRODUCTION

Citrus belongs to the family Rutaceae and is one of the important cash crops in the world (Swingle and Reece 1967). *Citrus* originated in Southeast Asia, which includes India, with a climate influenced by both east and south Asian monsoons (Jacques et al. 2011). Specific regions of its origin include the Yunnan province of southwest China (Gmitter and Hu 1990), Myanmar, and Northeastern India in the Himalayan foothills (Swingle and Reece 1967). The Northeastern part of India is a treasure house of *Citrus* species and their varieties (Sharma et al. 2004) where 11.7% of its total area is under its cultivation (NHB 2006). Mandarin, *Citrus reticulata* Blanco, is a major cash crop in India, accounting for around 41% of all *Citrus* fruits produced (Das et al. 2005). The Northeastern section of India is home to a variety of mandarin varieties and could be regarded as its place of origin (Ray and Deka 2000). Further, *C. reticulata* is considered to be one of the ancestral species that migrated from its centre of origin (Wu et al. 2018). Despite the region being the centre of origin, the bewilderment of *Citrus* grown there being natural remains, as most of the named species are hybrids propagated via cloning (Dorji and Yapwattanaphun 2011b). The lineages that gave rise to the majority of presently cultivated cultivars, on the other hand, have been lost in undocumented antiquity, and their identities are still debated (Swingle and Reece 1967; Spiegel-Roy and Goldschmidt 1996). Farmers of Sikkim and Darjeeling Himalayas are oblivious to the cultivars that their forefathers planted. As a result, Sikkim mandarin is sometimes mistaken for Darjeeling mandarin, and vice versa (Gurung et al. 2017). Besides, with monoembryonic and polyembryonic cultivars, as well as a vast range of interspecific hybrids, mandarins are the most diversified category of *Citrus* fruits (Hodgson 1967). As a result, there are several reports which suggest that *C. reticulata* Blanco comprises of ecotypes which include Darjeeling mandarin and Khasi mandarin (Das et al. 2004a), while there are certain reports that suggest *C. reticulata* comprise of Sikkim mandarin as well (Kishore et al. 2010).

Therefore, in order to clear this confusion, it is imperative to closely observe the morphological characters of the cultivated mandarin species and derive a relationship between them. There is a high possibility that domestication has led to changes in the attributes of present mandarins that are far different than that of its wild progenitors (Colunga-GarciaMarin et al. 1996). Furthermore, natural hybridization and spontaneous mutations are common phenomena in *Citrus* spp. (Dorji and Yapwattanaphun 2011a) which has led to the diversification of the species over the years. The evidence of the occurrence of zygotic twins (Das et al. 2007) attributes to greater variation and lack of uniformity in fruit quality in the plant (Dorji and Yapwattanaphun 2011a). The level of diversity that exists is unclear and a comprehensive study related to it has not been conducted in Darjeeling and Sikkim Himalayas.

To achieve this, morphological characters were used to identify accessions that are superior in quality. DIVA-GIS has been used to identify potential areas for the collection of mandarin germplasm to resolve the problem of lack of uniformity of fruit quality. This would ensure proper utilization of the available diversity. DIVA-GIS is a Geographic Information System that assists the plant genetic resources and biodiversity communities to map the range of distribution of species in which they are interested (Hijmans et al. 2002). DIVA-GIS has been used to identify areas of high diversity in wild potatoes (Hijmans et al. 2000), *Jatropha curcas* (Sunil et al. 2009), *Piper* (Parthasarathy et al. 2006). Mandarin ensures livelihood security to the farmers of the region whose marketability is determined by the increase in fruit size and the ratio of total soluble solids (TSS) to acidity ratio (Dorji and Yapwattanaphun 2011b). The identification of superior accessions could help in breeding programmes, ensure the quality of fruits that meet the demand of the market. Considering the heterogeneity that mandarins exhibit and its importance in the commerce of the region, the present study addresses the following questions: (i) Are Darjeeling mandarin and Sikkim mandarin ecotypes? (ii) Has there been variation in mandarins over the years? (iii) What is the state of morphological variation of mandarins in Darjeeling and Sikkim Himalayas?

4.2 MATERIALS AND METHODS

4.2.1 Collection of plant Samples

The sampling of fruits was executed from December 2017 to January 2018 and that of leaves and flowers in March of the same year. A total of 105 accessions were collected from four districts – North, South, East, and West districts of Sikkim and Darjeeling, Kalimpong districts of the state of West Bengal (WB), India (Fig. 8). Accession number and their sources are shown in Tables 5, 6. Each accession was recorded for leaves, flowers, and fruit characters. 5 flowers, 15 leaves, and 10 matured fruits were sampled randomly from each accession propagated through seedling. The coordinates and altitude of the orchard were recorded using Global Positioning System (Garmin GPS-12).

4.2.2 Data collection

Data for each accession was logged according to the International Plant Genetic Resource Institute description for citrus (IPGRI 1999). Data were recorded on fourteen quantitative and ten qualitative characters. Qualitative characters were measured based on coding while the fruit weight was measured using an electronic balance with 0.01 g precision. TSS was measured by a refractometer (Erma, Japan) and citric acid content was analysed by acid-base titration method. To evaluate the relationship between different quantitative characters, ANOVA was performed using Microsoft Excel 2007. Correlation coefficient (r) among the quantitative traits was evaluated using R package "corrplot" version 0.84 (Wei and Simko 2017).

4.2.3 Cluster and principal component analysis

The phenetic relationship between the accessions was evaluated by Principal Component Analysis (PCA) using the R package "Biodiversity R" (Kindt and Coe 2005). The relationship between accessions was interpreted through the construction of an unrooted Neighbor Joining (NJ) dendrogram based on a simple matching coefficient using Darwin version 6.0 (Perrier and Jacquemound-Collet 2006).

4.2.4 DIVA-GIS for diversity analysis

DIVA-GIS software version 7.2.1 (www.DIVAGIS.org) was used to generate grid maps for the analysis of the diversity of three important quantitative traits for marketability *viz.,* weight, diameter, and TSS/acidity recorded from various orchards coordinated with geographical coordinates. *The point-to-grid* option using a *circular neighborhood* method on the *"Analysis Menu"* was used to get grids over the points from where fruit samples were collected. Shannon diversity index was used to obtain the diversity patterns of the quantitative traits chosen.

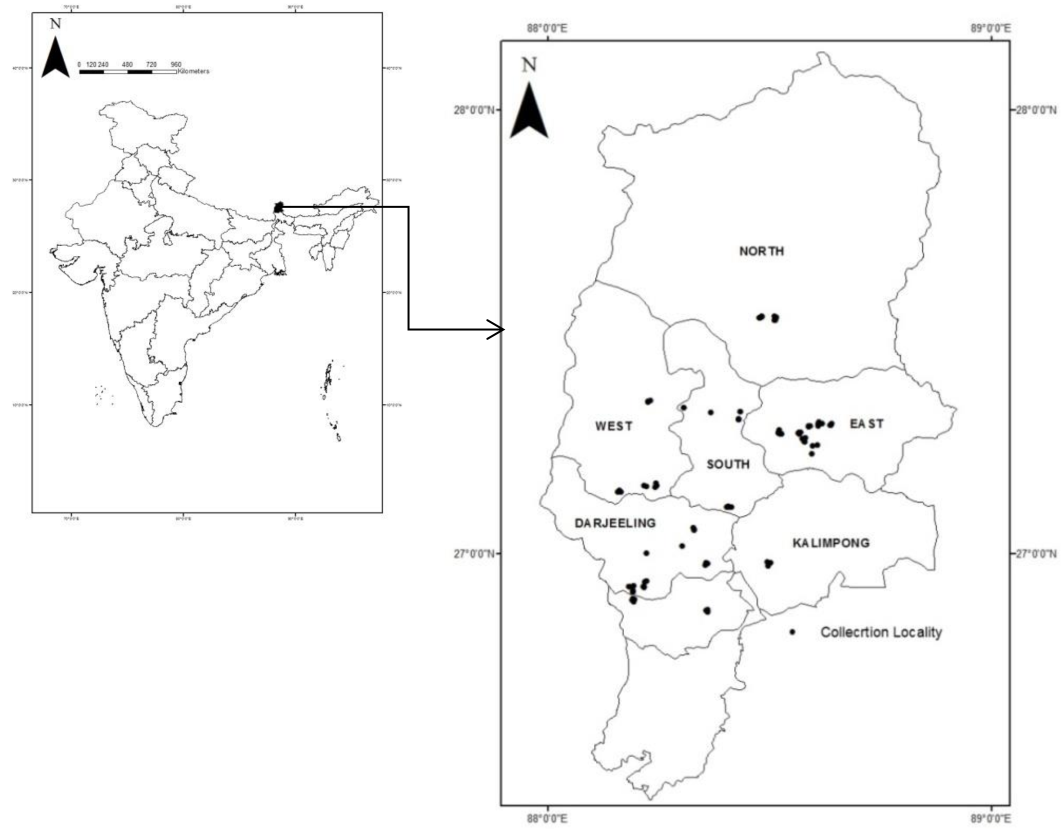


Fig. 8. Collection sites of *C. reticulata* from Sikkim and Darjeeling Himalayas

4.3 Results

4.3.1 Multivariate analysis of fruit descriptors

Multivariate analysis is an effective way to visualize and quantify divergence among populations due to variable traits. The eight fruit descriptors were further analysed for PCA to identify variables that contributed to maximum variance. PCA revealed five major principal components that contribute to the majority of the total cumulative variance. PC1 to PC5 contributed to a total of 88.4% variance (Fig. 9).

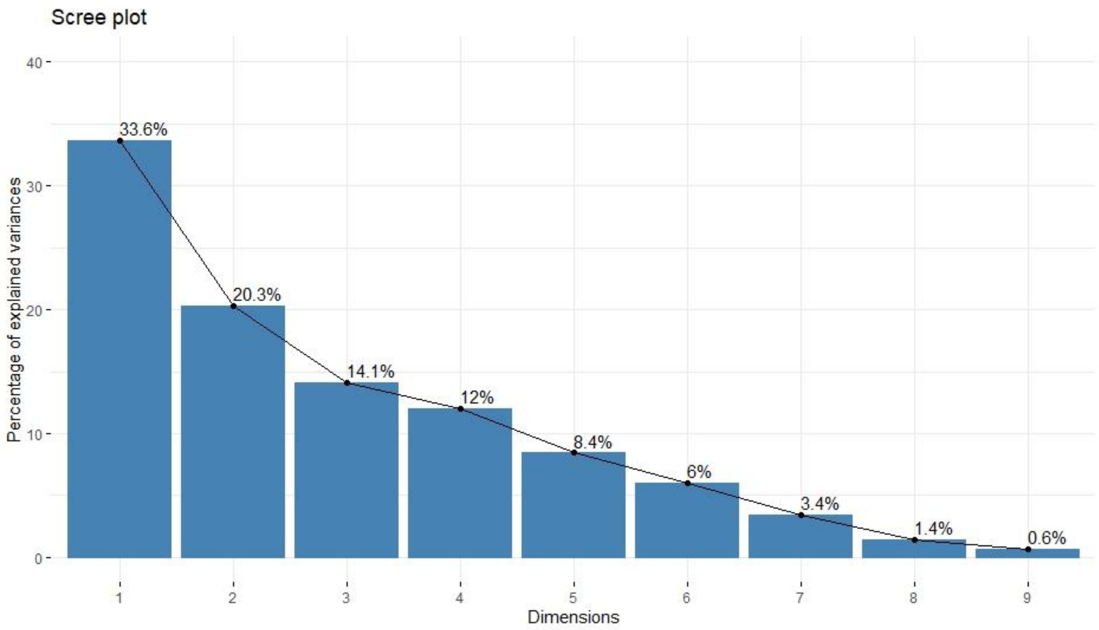


Fig. 9. Principal component analysis (PCA) scree plot illustrating percent variation by each principal component

All mandarin accessions were dispersed across all quadrants of the PCA ellipse plot and displayed no distinct clustering (Fig. 10). Accessions belonging to Darjeeling were largely found in the positive quadrant of PC1 while most accessions of Sikkim and Kalimpong were found in the positive quadrant of PC1 and PC2 and negative quadrant of PC1. In PC1 weight, height, diameter of fruit showed positive contribution while TSS and acidity contributed negatively. In PC2 epicarp width, height, weight, TSS, acidity has positive contributions while seeds and diameter have a negative contribution (Fig. 11). The diameter, number of seeds and TSS/Acidity of fruits contributed significantly to populating few of the Darjeeling accessions away from the other accessions (Fig. 10). Measurements such as height, weight, diameter, epicarp width, and number of seeds are in the positive quadrant and therefore accessions found in this quadrant are desirable for breeding desirable fruits.

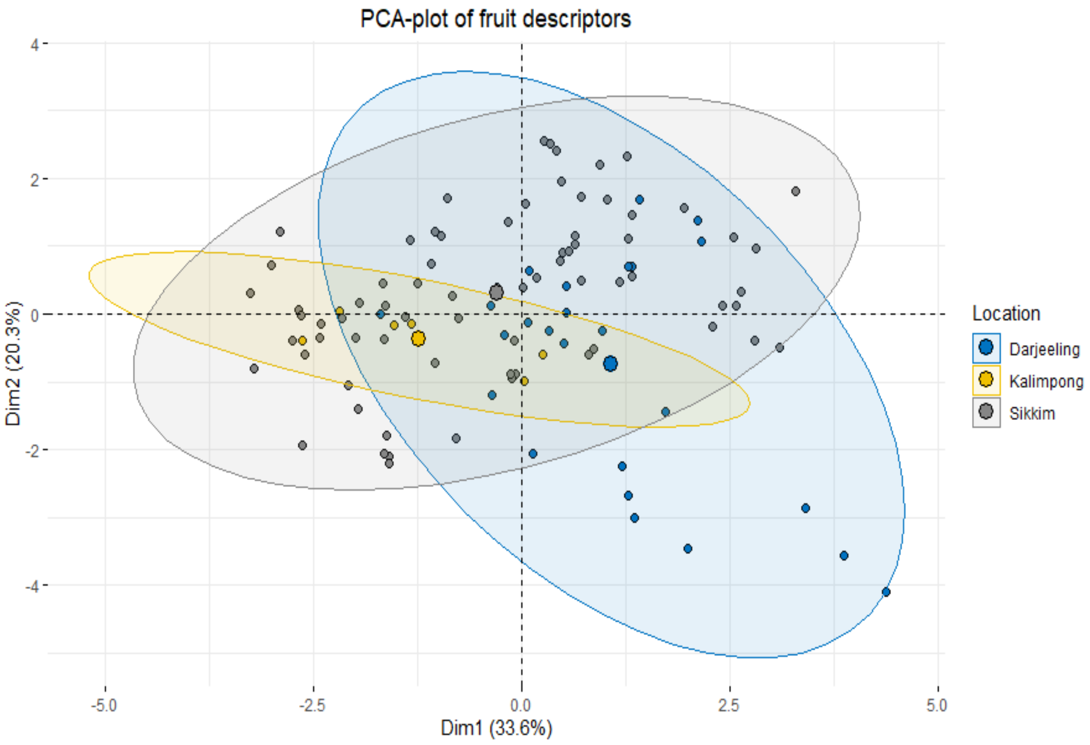


Fig. 10. PCA ellipse plot displaying clusters of mandarin accessions categorized by fruit attributes. Each ellipse represents accessions collected from different locations.

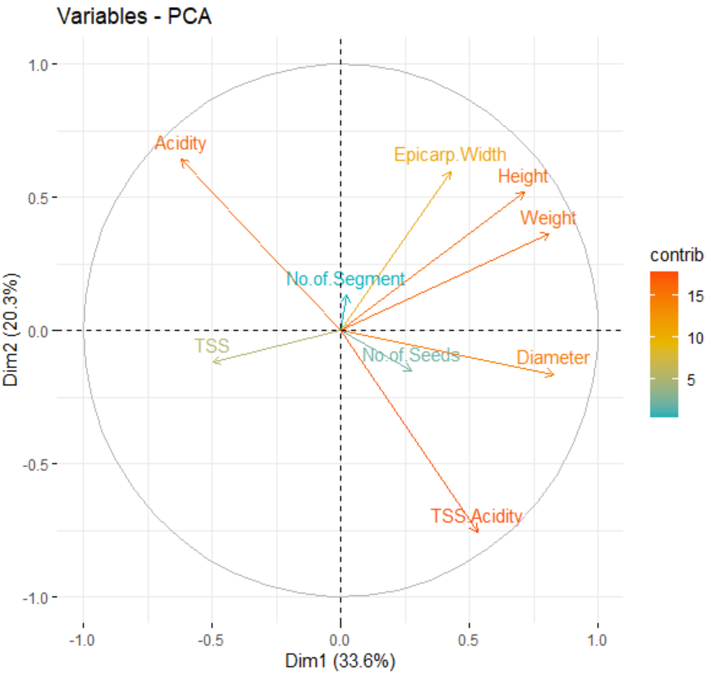


Fig. 11. PCA feature plot displaying contribution of fruit descriptor

4.3.2 Correlation network

The statistical analysis of quantitative characters showed high significant variations among the leaves, flowers, and fruits of different accessions except for the number of segments in a fruit and leaf length/width ratio (Table 2). S-02-08 (South district, Sikkim) had the longest leaf lamina length while D-02-05 (Kalimpong district, WB) had the lowest (Table 6). The broadest leaf was recorded in S-03-08 (East district, Sikkim) while the narrowest leaf was recorded in D-01-18 (Darjeeling District, WB). Similarly, the heaviest fruit was recorded in S-03-32 (East district) while S-01-02 (North district, Sikkim) had the lowest fruit weight (Table 5). The fruits in D-01-07 (Darjeeling district) were found to have the largest diameter while the fruits in S-01-01 (North district) had the smallest fruit diameter. The highest %TSS was recorded in S-01-11 (North district) while the highest acidity was recorded in S-02-11 (South district). The %TSS to acidity ratio was found to be highest in D-01-09 (Darjeeling District) while the lowest was recorded in S-02-11 (South district). The estimated correlation coefficient showed that TSS/acidity is positively correlated with the diameter of the fruit (0.473*), height is positively correlated with weight (0.772*), diameter is positively correlated with weight (0.549*), and the number of seeds showed a positive correlation with the diameter (0.423*) (Fig. 12).

Table 2: Result of ANOVA testing for differences in morphological traits of fruit, flower, leaves collected from different accessions

Traits	DF	P-Value	Significance
No. of segment	34	0.009114	NS
Fruit Weight	34	3.73E-20	***
Fruit Diameter	34	3.69E-14	***
Fruit Length	34	3.81E-15	***
Width of Epicarp	34	7.73E-16	***
No. of seeds	34	7.62E-06	***
TSS	34	2.59E-15	***
Acidity	34	2.23E-09	***
Leaf Length	34	5.39E-05	***
Leaf Width	34	8.79E-08	***
Leaf Length/Width	34	0.510286	NS
Petal Length	34	3.35E-12	***
Petal Width	34	2.14E-08	***
Pedicle Length	34	2.89E-14	***
No. of stamens	34	1.96E-24	***

The P-value of significant traits are in boldface (at P <0.001); NS, Non-significant

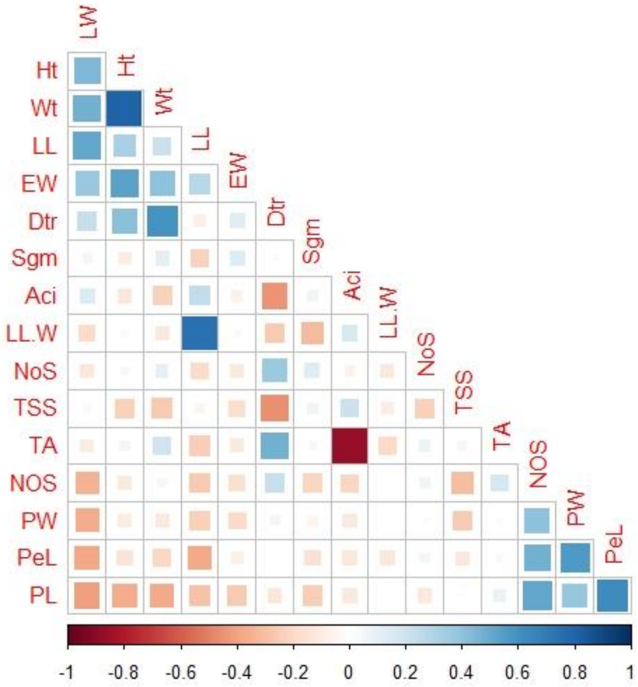


Fig. 12. Correlation coefficient among fourteen quantitative characters of *C. reticulata*. Abbreviation: Height (Ht), Weight (Wt), Diameter (Dtr), No. of seeds (NoS), Width of Epicarp (EW), TSS/Acidity (TA), No. of Segments (Sgm), Leaf Length (LL), Leaf Width (LW), Leaf Length/Width (LL. W), No. of Stamens (NOS), Pedicel Length (PeL), Petal Width (PW), Petal Length (PL)

4.3.3 Principal component analysis

PCA revealed that the six-axis contributed 78.40% to the total variation (Table 3; Fig. 13). After the inclusion of attributes of leaves and flowers along with the quantified attributes of fruits, we observed dispersion of accessions across all quadrants (Fig. 14) similar to that of Fig. 10. However, the majority of the accessions from Darjeeling populated in the positive quadrant of PC2, negative quadrant of PC1 and were relatively positioned distinctly than the accession from other locations. In PC1, TSS/Acidity, petal length, petal width, pedicel length, number of seeds contributed positively while fruit weight, segments, diameter, height, leaf length, width contributed negatively (Table 3; Fig. 15). In PC2, fruit and flower measurements contributed positively whereas leaf attributes contributed negatively (Table 3; Fig. 15). Related descriptors were found in the same quadrant whereas dissimilar descriptors were found in the opposite quadrants (Fig. 15). The attributes associated with flowers and TSS/Acidity largely contributed in characterizing Darjeeling accession whereas the attributes of fruits except TSS/Acidity and leaf contributed in characterizing accessions of Sikkim and Kalimpong (Fig. 15).

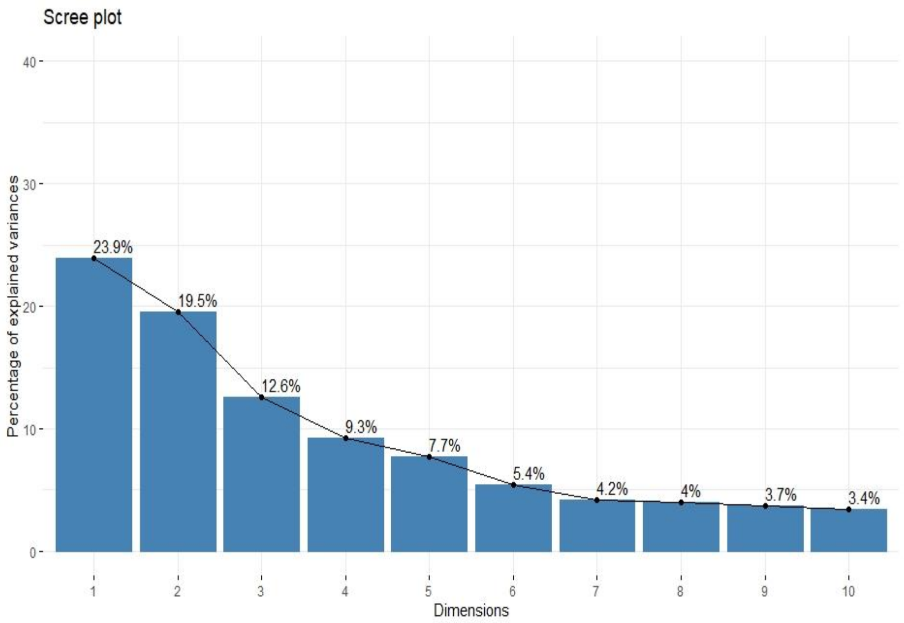


Fig. 13. Principal component analysis (PCA) scree plot illustrating percent variation by each principal component

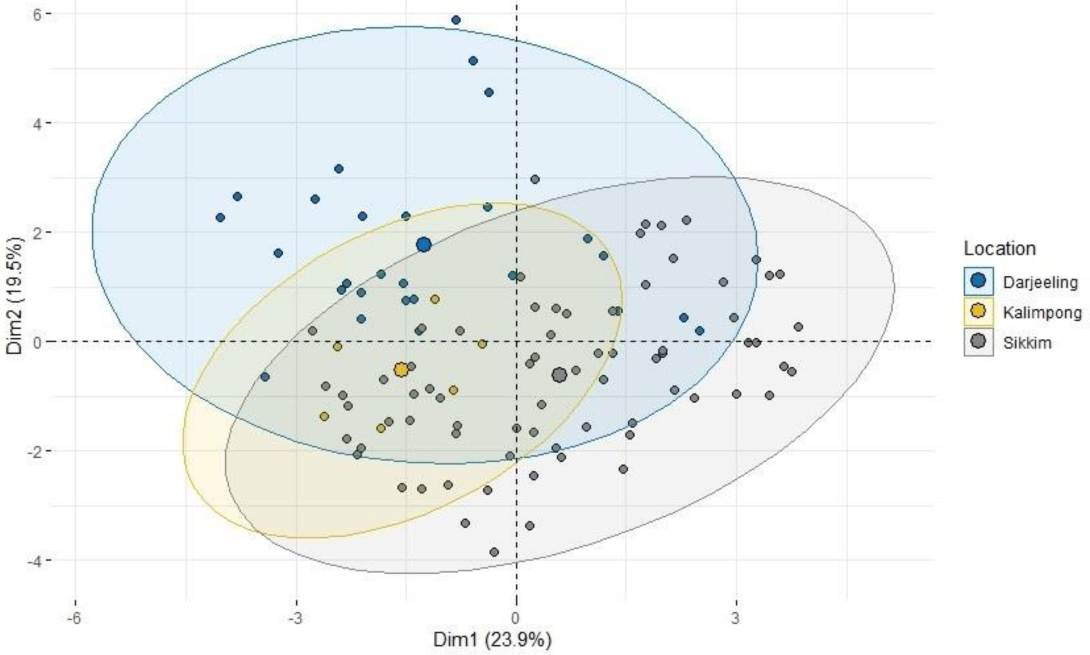


Fig. 14. Principal Component analysis depicting the contribution of 14 quantitative characters for variation among the 105 accessions of *C. reticulata*. The two components of principal component analysis (Dim1 and Dim2) are plotted on the axes

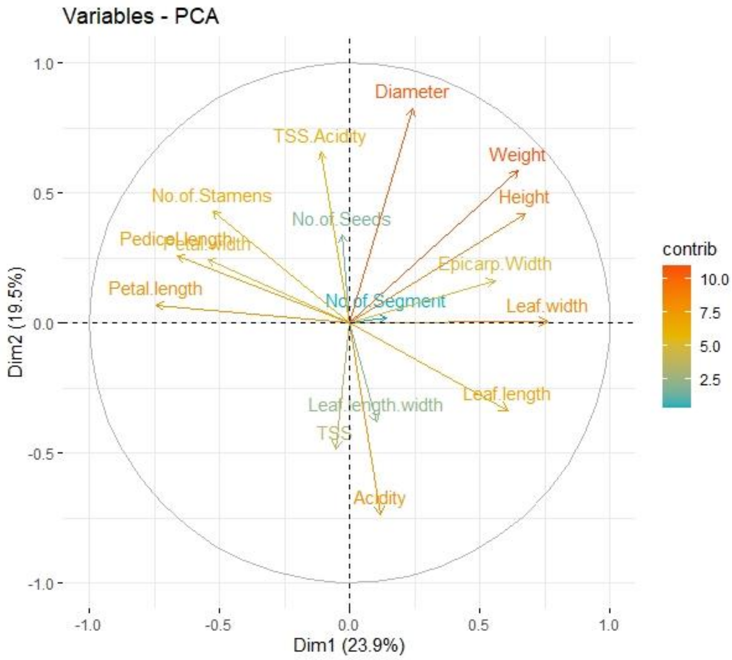


Fig. 15. PCA feature plot displaying contribution of fourteen quantitative characters among 105 accessions of *C. reticulata*

Table 3: Eigen values, proportion of variation and contribution of 14 quantitative characters to the total variation in the first six PC axes of the 105 accessions of *C. reticulata*.

Attributes	PC1	PC2	PC3	PC4	PC5	PC6	Total (%)
Sgm	-0.072	0.011	-0.412	0.337	-0.006	0.519	
Wt	-0.331	0.330	0.078	0.092	0.113	-0.092	
Dtr	-0.125	0.466	0.002	0.038	-0.174	-0.201	
Ht	-0.346	0.238	0.203	0.080	0.183	-0.046	
EW	-0.286	0.092	0.128	0.148	0.362	0.448	
NoS	0.014	0.190	-0.114	0.281	-0.610	-0.148	
TSS	0.026	-0.275	-0.313	-0.285	0.284	-0.179	
A	-0.060	-0.416	0.065	0.433	-0.019	-0.311	
TA	0.055	-0.275	-0.183	-0.542	-0.028	0.086	
LL	-0.310	-0.189	0.411	-0.180	-0.112	0.021	
LW	-0.388	0.002	-0.020	0.035	0.248	-0.380	
LL. W	-0.052	-0.215	0.496	-0.238	-0.323	0.302	
PL	0.380	0.039	0.187	-0.056	0.257	-0.125	
PW	0.268	0.137	0.237	0.252	0.085	0.180	
NOS	0.268	0.242	0.268	0.060	0.032	-0.181	
PeL	0.338	0.146	0.190	0.207	0.289	-0.004	
Eigen Value	3.824	3.120	2.016	1.482	1.231	0.870	
Variance (%)	23.90	19.50	12.60	9.26	7.69	5.43	**78.40**

P.C: Principal Component

4.3.4 Cluster analysis

Cluster analysis was utilized to identify distinct clusters based on germplasm classification patterns. A dendrogram generated from unrooted neighbor-joining method for qualitative and quantitative characters of 105 accessions classified it into two major clusters (cluster I and cluster II; Fig. 16). The addition of qualitative characters (Table 4) in the analysis has resulted in the distinct separation of accessions from Darjeeling and Sikkim (Plate 1-6). Further, the accessions from Darjeeling and east district formed a distinct sub-cluster while the other accessions clustered with each other irrespective of their geographical association. Overall, the morphological qualitative and quantitative parameters for the accessions diverged at a similarity coefficient of 0.49.

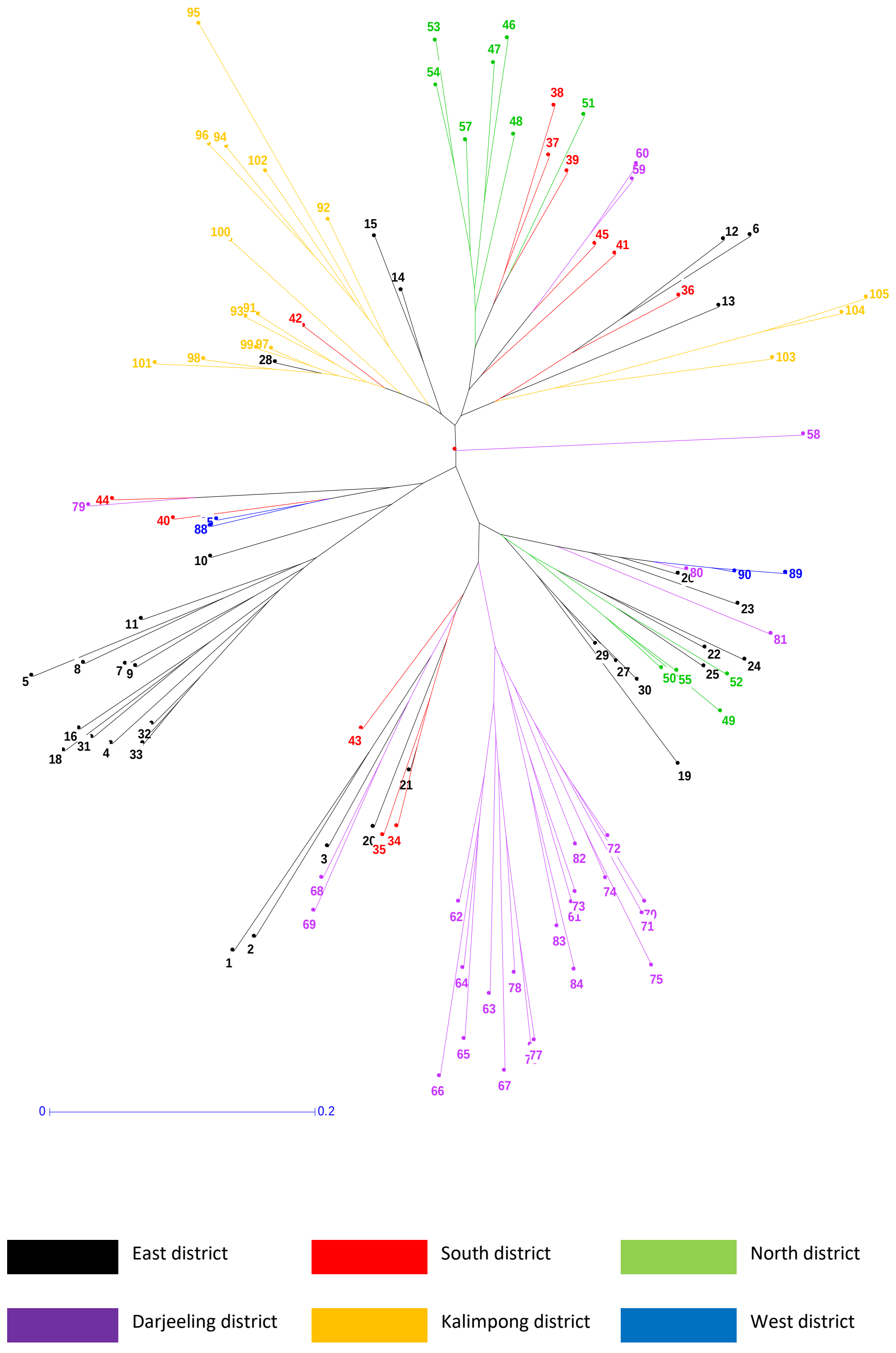


Fig. 16. Unrooted Neighbour-joining tree depicting relationship among the 105 accessions of *C. reticulata* based on qualitative and quantitative variables of trees, leaves, flowers and fruits. Different colour legends in the figure depict collection sites of 105 *C. reticulata* accessions from Sikkim and Darjeeling Himalayas

Table 4: Frequency distribution for the measure of qualitative characters in mandarin (*C. reticulata*) accessions

Character	**Frequency (%)**
Adherence of segment walls	Strong (8), Medium (87), Weak (5)
Segment Uniform	Yes (8), No (92)
Fruit Shape	Obloid (82), Spheroid (18)
Shape of fruit apex	Depressed (10), Truncate (88), Concave (1), Convex (1)
Shape of fruit base	Depressed (2), Truncate (84), Concave (6), Convex (7), Spheroid (1)
Seed Shape	Ovoid (9), Clavate (91)
Intensity of green color	Medium (47), Dark (53)
Leaf lamina shape	Elliptic (98), Lanceolate (2)
Leaf lamina margin	Entire (66), Sinuate (34)
Petiole width	Narrow (50), Medium (50)
Relative length of stamen to stigma	Medium (52), Short (48)

4.3.5 Grid-Mapping

The highest diversity index for TSS/acidity, fruit weight, and diameter were observed in the East district, Sikkim (Fig. 17, 18, 19). The highest coefficient of variation in fruit weight was observed in Darjeeling district, WB and East district, Sikkim (Fig. 19) and TSS/acidity and fruit diameter in Darjeeling district, WB (Fig. 17, 18).

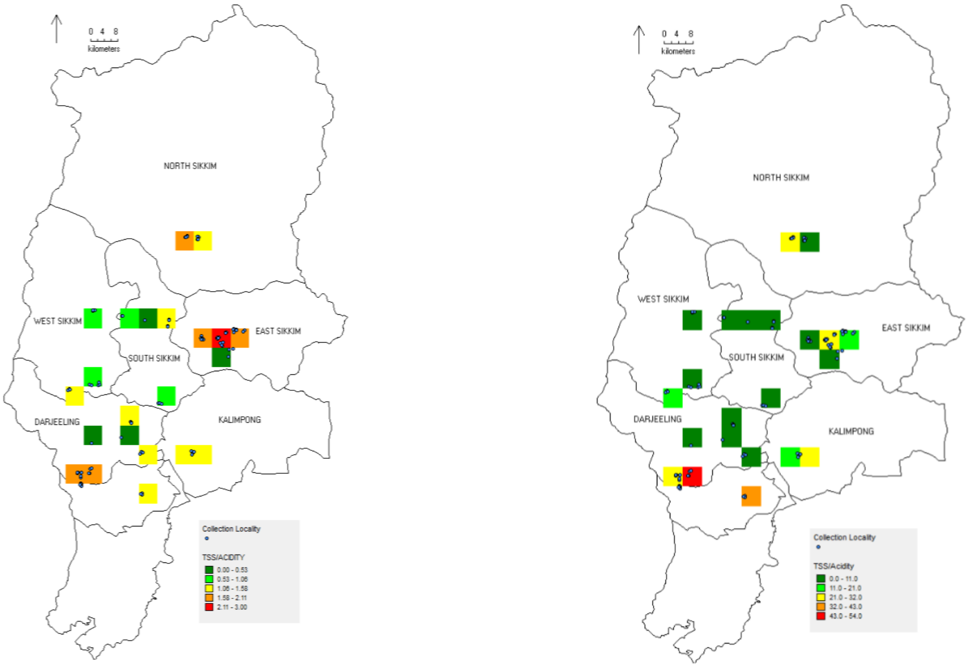


Fig. 17. Grid map showing diversity index and coefficient of variation for TSS/Acidity of *C. reticulata*

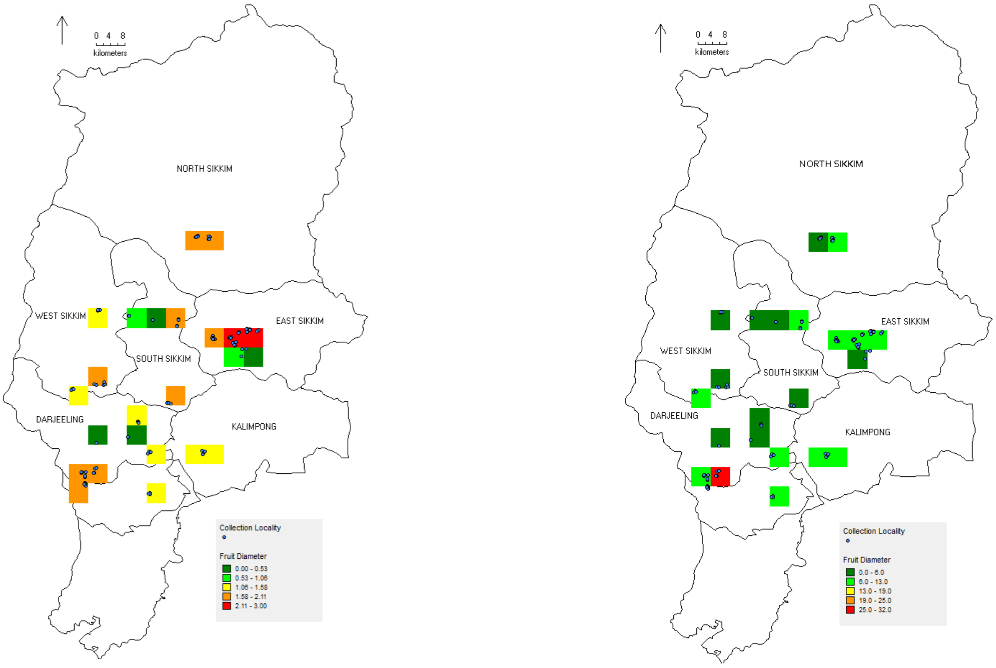


Fig. 18. Grid map showing diversity index and coefficient of variation for fruit diameter of *C. reticulata*

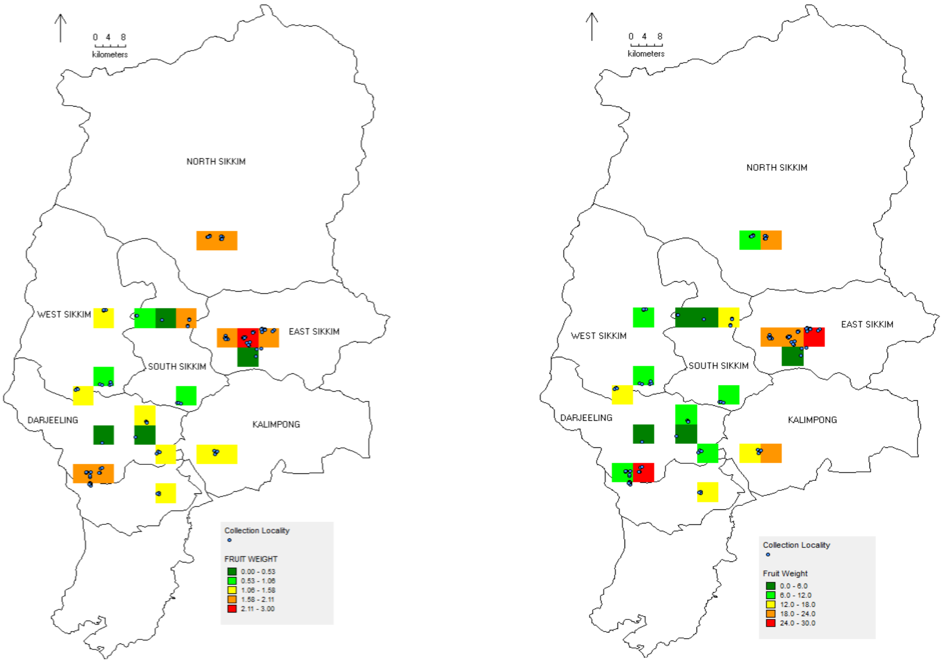


Fig. 19. Grid map showing diversity index and coefficient of variation for fruit weight of *C. reticulata*

4.4 Discussion

The study of phenotypic diversity based on morphological characters is less favoured due to the morphological plasticity that it exhibits (Dorji and Yapwattanaphun 2011b). However, several studies that include both morphological and molecular markers have suggested them to be independent of each other (Koehler et al. 2003; Campos et al. 2005). Furthermore, studies have reported that multiple genes control the agronomic traits and the ideal way to assess it is through morphological means (Liu and Deng 2007).

In the present work, we examined the morphological diversity of mandarin with the assistance of the qualitative and quantitative characters of fruits, leaves, and flowers. By examining the vegetative and fruit descriptors, we found wide variation in the accessions under scrutiny. The phenotypic diversity of mandarins of Darjeeling and

Sikkim Himalayas is apparent and is in accordance with the result published on mandarin morphological diversity of Bhutan by Dorji and Yapwattanaphun (2011a).

GIS mapping helps in predicting distribution, documentation, analysing diversity and variation, developing new strategies for conservation (Abraham et al. 2010). It has also become an important tool to visually elucidate the diverse nature of a species and identify its location (Flemons et al. 2007). As anticipated, the grid maps generated through DIVA-GIS showed wide variations and diversity within and between the districts in our present study which suggests that environmental factors alone do not trigger morphological variation. Cross-pollination, propagation through seed without nucellar selection and mutation has produced a large variation in plant type and fruit quality (Dorji and Yapwattanaphun 2011a; 2011b) and is apparent among the plants in the same orchard (Das et al. 2007). The highest diversity index and coefficient of variation for characters that are pertinent for marketability were observed for the East and Darjeeling districts, indicating the presence of diverse accessions.

The TSS content for the analysed accessions varied significantly among all the accessions. The level of TSS/acidity indicates the sweetness of an orange (Kishore et al. 2010) which is directly proportional to each other. We found that the accessions from D-01-09 (Darjeeling district) had the highest TSS/acidity as compared to the other accessions. The correlation coefficient between TSS/Acidity, diameter (0.48*), height, weight (0.80*), and diameter, weight (0.59*) showed a positive correlation, which is similar to the findings by Gill et al (2005).

The application of nitrogen fertilizers (N) has been shown to contribute positively towards % TSS, juice, and acid content (Zekri et al. 2009). However, Thompson et al (2005) and Kusakabe et al (2006) found that nitrogen rates and fertilizers did not significantly affect the fruit quality including the percentage of TSS and acid content. Since, the use of fertilizers has been banned in the state of Sikkim and the farmers of Darjeeling and Kalimpong have been practicing organic farming, the variation in TSS could be due to genetic variation. The aim of *Citrus* breeders over the past 30 years is to develop seedless, easy-peeling, enriched flavour and aroma and extension of ripening period varieties (Deng 2005). Various methods are being designed to develop seedless varieties (Bilinques 2004; Liu and Deng 2007). The inherited seedless trait exhibited continuous variation over generations (Liu and Deng 2007). Nevertheless, S-03-18 (East Sikkim) which contained the lowest number of seeds per fruit can be used for the multiplication of seedless varieties.

PCA indicated that the fruit descriptors except TSS/Acidity characterized Sikkim mandarin while the flower descriptors including TSS/Acidity characterized Darjeeling mandarin. The non-uniformity of fruit quality and maturation across the growing locations may be related to genetic variation as well as geographic and environmental factors (Dorji and Yapwattanaphun 2011a). Therefore, fruit and flower characteristics can be a determinant factor in selecting superior varieties that can act as parents for breeding purposes. PCA also positioned the accession into separate quadrants collected from different locations which were governed by dissimilar descriptors. Cluster analysis based on qualitative and quantitative characters of 105 accessions divided them into two major clusters (I and II) and subsequent divergence to sub-clusters, which is in accordance with the findings in Bhutanese mandarin (Dorji and Yapwattanaphun 2011b; 2015). Further, the average similarity coefficient (0.49) indicated a wide variation in the morphological traits of the collected accessions. The accessions from Darjeeling formed a separate sub-cluster than the other sub-clusters which indicates that Sikkim and Darjeeling mandarin are ecotypes as suggested by Kishore et al (2010). The separate sub-clustering of accessions of east district respectively could be due to varied environmental conditions and genetic heterogeneity whereas the sub-clusters with mixed accession could be due to the similar genetic make-up of the species (Campos et al. 2005; Das et al. 2005) due to their ability to hybridize (Dorji and Yapwattanaphun 2011a). This could be because most of the trees planted by farmers are grown from seeds of diverse and unknown origins (Dorji and Yapwattanaphun 2015).

Table 5: Mean data of quantitative characters of fruits of 105 *C. reticulata* accessions

Accessions	**No. of Segments**	**Weight (g)**	**Diameter**	**Length (mm)**	**Epicarp width (mm)**	**No. of Seeds**	**TSS/Acidity**	**District**	**State**
D-01-01	9.7	79.78	62.4	50.79	2.31	15.20	14.42	Darjeeling	West Bengal
D-01-02	9.1	73.87	59.31	53.05	2.59	14.20	16.90	Darjeeling	West Bengal
D-01-03	9.1	73.87	59.31	53.05	2.59	14.20	16.90	Darjeeling	West Bengal
D-01-04	8.9	68.38	52.43	50.03	1.20	13.1	22.80	Darjeeling	West Bengal
D-01-05	9.6	77	64.74	45.26	1.19	16.7	25.72	Darjeeling	West Bengal
D-01-06	8.9	68.85	75.20	45.08	1.92	15.1	19.26	Darjeeling	West Bengal
D-01-07	9.7	106.20	110.10	39.09	1.34	15.6	33.56	Darjeeling	West Bengal
D-01-08	8.6	78.55	123	42.08	1.29	18.5	32.93	Darjeeling	West Bengal
D-01-09	9.7	118.30	74.80	47.57	1.31	17.7	36.09	Darjeeling	West Bengal
D-01-10	9.9	97.5	71.92	54.05	2.22	16.10	11.69	Darjeeling	West Bengal
D-01-11	9.6	89.49	70.95	50.79	2.35	12.70	9.72	Darjeeling	West Bengal
D-01-12	9.7	96.24	71.84	52.83	2.14	18	11.62	Darjeeling	West Bengal
D-01-13	8.1	71.04	64.58	46.07	1.18	15.4	21.64	Darjeeling	West Bengal
D-01-14	9.4	75.35	66.06	45.98	1.05	11.9	31.37	Darjeeling	West Bengal
D-01-15	9.4	76.87	66.48	46.07	1.18	15.4	36.07	Darjeeling	West Bengal
D-01-16	9.2	62.21	62.55	45.61	1.5	13.2	10.32	Darjeeling	West Bengal
D-01-17	9	72.73	59.94	46.41	1.43	11.3	12.85	Darjeeling	West Bengal
D-01-18	9.9	88.37	72.43	44.56	1.45	11.8	12.64	Darjeeling	West Bengal
D-01-19	8.8	48.36	48.25	45.47	2.18	15.6	12.40	Darjeeling	West Bengal
D-01-20	8.3	58.03	61.78	48.94	2.08	14.5	10.26	Darjeeling	West Bengal
D-01-21	8	54.23	57.35	45.47	2.19	12.5	10.98	Darjeeling	West Bengal
D-01-22	9.5	68.46	53.24	43.86	1.76	8.4	22.40	Darjeeling	West Bengal
D-01-23	9.6	55.93	46.38	40.68	1.71	11.6	11.42	Darjeeling	West Bengal
D-01-24	9.4	73.01	54.61	44.24	1.70	15	13.74	Darjeeling	West Bengal
D-01-25	9.5	68.12	62.47	41.93	2.01	11.8	14.19	Darjeeling	West Bengal

D-01-26	8.5	58.8	56.71	49.31	2.25	9.8	16.65	Darjeeling	West Bengal
D-01-27	8.5	60.67	58.37	50.46	2.2	10	14.08	Darjeeling	West Bengal
D-02-01	9	76.45	57.09	47.28	1.64	12.5	16.87	Kalimpong	West Bengal
D-02-02	9.5	55.98	48.76	43.38	1.65	12.6	12.34	Kalimpong	West Bengal
D-02-03	9.2	60.40	47.63	41.44	1.76	11.9	12.74	Kalimpong	West Bengal
D-02-04	9.4	74.54	53.87	44.98	1.65	14.6	18.79	Kalimpong	West Bengal
D-02-05	9.4	49.81	45.46	37.98	1.56	12.2	11.68	Kalimpong	West Bengal
D-02-06	9.4	57.57	44.71	39.86	1.7	13.2	11.10	Kalimpong	West Bengal
S-01-01	8.66	49.1	43.37	41.29	1.6	8	17.74	North	Sikkim
S-01-02	8.55	37.91	44.31	37.02	1.49	4.4	20.33	North	Sikkim
S-01-03	9.4	48.99	43.41	41.49	1.51	8.1	22.70	North	Sikkim
S-01-04	9.1	52.84	50.01	41.52	1.91	12.9	15.37	North	Sikkim
S-01-05	8.9	46.21	47.4	36.89	1.31	6.7	20.36	North	Sikkim
S-01-06	9.1	47.32	47.86	36.89	1.31	6.6	20.06	North	Sikkim
S-01-07	8.9	63.66	51.85	45.64	1.96	11.8	20.16	North	Sikkim
S-01-08	9	63.84	51.38	45.29	1.9	11.7	20.22	North	Sikkim
S-01-09	9	63.7	51.24	45.37	1.94	11.9	20.16	North	Sikkim
S-01-10	9.6	44.95	48.25	40.25	1.8	17.16	12.22	North	Sikkim
S-01-11	9.8	39.74	43.55	37.79	1.6	18.1	13.28	North	Sikkim
S-01-12	11.4	48.13	47.98	38.33	1.75	11.4	13.37	North	Sikkim
S-02-01	9.7	89.69	57.86	48.61	1.47	16.1	16.37	South	Sikkim
S-02-02	10.2	92.87	56.35	46.43	1.77	12.9	13.89	South	Sikkim
S-02-03	9.1	88.73	57.8	49.05	1.47	16	16.33	South	Sikkim
S-02-04	8.7	63.34	53.24	45.83	1.1	11.8	12.48	South	Sikkim
S-02-05	8.5	49.22	45	43.55	1.28	12.5	8.65	South	Sikkim
S-02-06	8.7	52.11	47.42	41.54	1.63	12.2	11.92	South	Sikkim
S-02-07	9.2	103.89	55.74	48.65	2.27	11.7	16.85	South	Sikkim
S-02-08	10	118.4	65.81	50.16	2.54	9.2	14.89	South	Sikkim
S-02-09	9.7	108.72	63.74	51.68	2.23	9.3	13.24	South	Sikkim
S-02-10	8.8	72.72	50.98	48.72	1.58	16.4	7.64	South	Sikkim
S-02-11	9.8	50.97	66.65	46.1	1.43	14.4	6.55	South	Sikkim

S-02-12	9.6	45.05	42.23	37.31	1.31	12.6	7.62	South	Sikkim
S-03-01	9.4	121.13	69.53	57.56	3.39	17.13	13.47	East	Sikkim
S-03-02	9.7	113.14	61.35	52.46	2.64	15.3	14.14	East	Sikkim
S-03-03	9.8	85.75	57.65	49.01	2.22	8.2	16.74	East	Sikkim
S-03-04	9.2	97.23	57.91	53.13	1.9	13.9	11.79	East	Sikkim
S-03-04	9.8	99.98	60.16	54.56	2.49	6.2	7.26	East	Sikkim
S-03-05	9.4	98.82	57.21	49.61	2.05	12.5	14.72	East	Sikkim
S-03-06	9.9	104.65	58	51.69	2.25	12.5	14.88	East	Sikkim
S-03-07	8.7	91.41	58.99	58.99	1.97	8.6	8.78	East	Sikkim
S-03-08	9.2	102.30	60.02	59.01	1.72	12.4	10.51	East	Sikkim
S-03-09	9	93.76	56.91	53.34	1.68	13.5	10.69	East	Sikkim
S-03-10	9.27	90.66	60.21	54.87	2.03	9.81	7.37	East	Sikkim
S-03-11	9.77	98.04	55.08	49.08	1.93	10.77	13.07	East	Sikkim
S-03-12	10.4	97.18	58.88	53.06	2.3	16.6	9.8	East	Sikkim
S-03-13	9.8	91.34	40.97	35.63	1.41	12.1	11.80	East	Sikkim
S-03-14	9.6	78.91	55.53	45.48	1.96	12.5	12.07	East	Sikkim
S-03-15	9.4	109.93	62.37	51.18	2.14	7.4	8.75	East	Sikkim
S-03-16	9	104.29	57.81	54.52	1.79	10.6	10.93	East	Sikkim
S-03-17	9	121.22	62.28	52.71	2.29	15	9.23	East	Sikkim
S-03-18	9.3	52.17	46.17	42.95	1.66	8.1	8.00	East	Sikkim
S-03-19	10.1	97.21	55.96	48.59	1.83	12.8	10.37	East	Sikkim
S-03-20	9.6	102	58.83	49.01	1.92	13.5	14.59	East	Sikkim
S-03-21	9.5	55.03	50.1	40.6	2.38	9	8.87	East	Sikkim
S-03-22	9.7	54.28	47.44	39.58	1.76	11.4	11.03	East	Sikkim
S-03-23	9	58.42	47.69	37.43	2.33	9.4	11.13	East	Sikkim
S-03-24	9.3	45.04	47.09	38.78	1.51	9.7	14.82	East	Sikkim
S-03-25	9.6	42.66	44.71	36.55	1.52	9.2	10.95	East	Sikkim
S-03-26	8.6	50.58	46.08	40.11	1.59	9.2	10.65	East	Sikkim
S-03-27	8.5	48.42	45.29	37.96	1.37	8.6	15.23	East	Sikkim
S-03-28	9.6	42.66	44.71	36.55	1.52	9.2	11.88	East	Sikkim
S-03-29	8.6	50.58	46.08	40.11	1.59	9.2	12.07	East	Sikkim

S-03-30	8.8	105.45	62.13	52.33	1.97	8.5	9.22	East	Sikkim
S-03-31	9.2	103.03	56.32	54.32	1.5	10	10.78	East	Sikkim
S-03-32	9.1	122.16	62.46	52.47	1.72	11.4	9.79	East	Sikkim
S-04-01	9.3	60.69	49.33	39.77	1.59	8.9	9.98	West	Sikkim
S-04-02	9.7	70.3	54.61	44.08	1.62	10.8	11.26	West	Sikkim
S-04-03	9.6	65.63	49.23	41.62	1.98	10.7	9.89	West	Sikkim
S-04-04	9.8	78.35	57.21	50.03	2.07	12.3	14.84	West	Sikkim
S-04-05	10.2	80.33	58.62	45.26	2.09	15.1	12.98	West	Sikkim
S-04-06	9.8	68.6	55.04	45.08	2.14	13.3	7.81	West	Sikkim
S-04-07	10	48.51	46.25	38.16	1.56	13.3	7.73	West	Sikkim
S-04-08	9.3	58.91	49.02	40.01	1.8	13.7	9.15	West	Sikkim
S-04-09	9.4	56.38	46.41	38.15	1.62	11.2	9.17	West	Sikkim
S-04-10	11.4	82.43	61.74	50.51	2.97	10.7	19.88	West	Sikkim
S-04-11	9	114.75	64.46	55.87	3	11.8	16.72	West	Sikkim
S-04-12	8.9	100.35	64.71	53.98	2.61	10.7	17.70	West	Sikkim
S-04-13	9.3	72.4	55.16	45	1.82	8.3	15.28	West	Sikkim
S-04-14	9.4	54.56	47.55	40.48	1.61	9.9	11.48	West	Sikkim
S-04-15	9.4	71.89	51.68	44.24	1.89	12.7	16.38	West	Sikkim

Table 6: Mean data of quantitative characters of leaf and flower of 105 *C. reticulata* accessions

Accessions	**Leaf Length (mm)**	**Leaf width (mm)**	**Leaf length/width**	**Petal length**	**Petal width**	**No. of Stamens**	**Pedicel Length**	**District**	**State**
D-01-01	86.87	35.93	2.41	9.2	3.4	13.8	2.8	Darjeeling	West Bengal
D-01-02	86.87	35.93	2.41	9.2	3.4	13.2	2.8	Darjeeling	West Bengal
D-01-03	74.00	29.87	2.47	9.2	3.4	14.4	2.8	Darjeeling	West Bengal
D-01-04	68.00	31.07	2.25	10.4	4.4	16.8	3.2	Darjeeling	West Bengal
D-01-05	68.73	30.13	2.28	10.2	3.8	16.4	3.2	Darjeeling	West Bengal
D-01-06	76.07	35.33	33.8	10	3.8	16.8	3.3	Darjeeling	West Bengal
D-01-07	70.46	31.13	2.26	10.8	4.4	16.8	3.2	Darjeeling	West Bengal
D-01-08	61.73	28.06	2.19	10.6	3.2	16.4	3.6	Darjeeling	West Bengal
D-01-09	61.73	28.07	2.19	10.4	3.6	16.6	3.2	Darjeeling	West Bengal
D-01-10	79.46	34.06	2.33	10.1	4.8	13.6	4.2	Darjeeling	West Bengal
D-01-11	76.46	31.73	2.4	10.5	4.5	14.4	5.3	Darjeeling	West Bengal
D-01-12	79.47	34.07	2.33	9.5	4.6	14	4.9	Darjeeling	West Bengal
D-01-13	79.93	33.13	2.41	10.6	4.8	16.4	5	Darjeeling	West Bengal
D-01-14	71.26	28.13	2.53	10.6	5	16.4	5	Darjeeling	West Bengal
D-01-15	68.86	26.93	2.55	10	4.8	16	5	Darjeeling	West Bengal
D-01-16	80.86	34.4	2.35	11.2	4.8	16.4	5	Darjeeling	West Bengal
D-01-17	72.40	30.66	2.36	11.4	4.8	16.8	5.2	Darjeeling	West Bengal
D-01-18	48.93	22.47	2.17	10.2	4.6	15.6	5	Darjeeling	West Bengal
D-01-19	77.40	32.47	2.38	11.2	4.8	16.4	5	Darjeeling	West Bengal
D-01-20	73.06	38	1.92	11.4	4.8	16.4	5.2	Darjeeling	West Bengal
D-01-21	77.40	32.4	2.38	10.2	4.6	15.8	5	Darjeeling	West Bengal
D-01-22	68.60	34.66	1.97	11.2	4.1	16.4	5	Darjeeling	West Bengal
D-01-23	75.60	30.8	2.45	12.6	4.6	16.4	5.2	Darjeeling	West Bengal
D-01-24	63.27	31.07	2.03	10.6	4.8	16.6	5	Darjeeling	West Bengal
D-01-25	89.40	33.2	2.69	11.4	4.1	16.4	5	Darjeeling	West Bengal

D-01-26	83.73	31.66	2.64	13	4.6	16.4	4.9	Darjeeling	West Bengal
D-01-27	87.60	32.26	2.71	11.2	4.8	16.6	5.1	Darjeeling	West Bengal
D-02-01	88.66	34.8	2.54	11.4	3.6	14.4	3.6	Kalimpong	West Bengal
D-02-02	63.07	34.6	1.82	11.4	4.4	14.8	4.6	Kalimpong	West Bengal
D-02-03	66	31.26	2.11	11.2	3.8	14.4	3.8	Kalimpong	West Bengal
D-02-04	63.80	32.8	2.54	11.4	3.6	14.4	3.6	Kalimpong	West Bengal
D-02-05	52.26	30.8	1.82	11	4	14.8	4.6	Kalimpong	West Bengal
D-02-06	60.78	32.41	2.11	9.6	3.4	14.4	3.8	Kalimpong	West Bengal
S-01-01	81.73	31.33	2.6	10.6	5.2	14.2	3.6	North	Sikkim
S-01-02	89.20	34.66	2.57	10.2	4.4	13.6	3.4	North	Sikkim
S-01-03	89.33	36.53	2.44	9.8	3.4	13.6	2.8	North	Sikkim
S-01-04	85.53	35.73	2.39	10.8	5	14.2	4.6	North	Sikkim
S-01-05	93.60	37.4	2.5	10.4	3.8	14.2	3.2	North	Sikkim
S-01-06	85.46	32.93	2.59	11.4	3.6	14	3.6	North	Sikkim
S-01-07	72.60	29.93	2.42	10.8	5	14.2	3.6	North	Sikkim
S-01-08	67.00	29.73	2.25	10.4	4.4	13.6	2.4	North	Sikkim
S-01-09	71.46	30.2	2.36	11.4	3.4	13.6	2.8	North	Sikkim
S-01-10	81.86	33.06	2.47	10.8	5.6	14.2	4.6	North	Sikkim
S-01-11	85.13	34.66	2.45	10.4	4	13.4	3.2	North	Sikkim
S-01-12	81.93	35	2.34	10.2	3.6	13.8	3.2	North	Sikkim
S-02-01	80.66	34.06	2.36	10.6	4.6	14	4.4	South	Sikkim
S-02-02	78.20	31.93	2.44	10.2	4.6	13.4	3.8	South	Sikkim
S-02-03	81.20	33.66	2.41	10.8	3.8	13.6	3.6	South	Sikkim
S-02-04	84.33	33.73	2.5	9.8	3.4	13.8	2.8	South	Sikkim
S-02-05	71.60	30	2.38	9.6	3.4	13.4	2.8	South	Sikkim
S-02-06	78.53	30.26	2.59	9.6	3.4	14	2.8	South	Sikkim
S-02-07	83.66	32.8	2.55	11.2	5.4	13.8	4.6	South	Sikkim
S-02-08	125.46	32.53	3.85	10.8	4.6	14.4	3.4	South	Sikkim
S-02-09	85.06	34.6	2.45	11.2	3.6	13.8	3.5	South	Sikkim
S-02-10	82.66	37.33	2.21	9.8	3.4	13.8	2.8	South	Sikkim
S-02-11	80.13	31.66	2.53	9.6	3.4	13.4	2.8	South	Sikkim

S-02-12	76.20	31.93	2.38	9.6	3.4	14	2.8	South	Sikkim
S-03-01	86	36.67	2.42	11.4	4.4	16	4.6	East	Sikkim
S-03-02	92.93	35.53	2.34	10.6	4.8	15.6	3.4	East	Sikkim
S-03-03	85.13	36.33	2.35	9.6	4.8	15.6	3	East	Sikkim
S-03-04	98.60	37.93	2.59	9.2	3.4	13.6	4.6	East	Sikkim
S-03-04	96.66	42.33	2.28	9.2	3.4	13	3.4	East	Sikkim
S-03-05	90.83	42.13	2.15	9.2	3.4	14	3	East	Sikkim
S-03-06	99.53	38.73	2.56	9.2	3.4	13.8	2.8	East	Sikkim
S-03-07	96.46	42.4	2.24	9.2	3.4	13.2	2.8	East	Sikkim
S-03-08	95.20	67.4	1.41	9.2	3.4	14.4	2.8	East	Sikkim
S-03-09	85.06	35.8	2.37	9	3.6	13.6	3.2	East	Sikkim
S-03-10	92.93	42.4	2.19	9.4	3.2	13	3	East	Sikkim
S-03-11	80.46	36.33	2.21	9.4	3.6	13	3.4	East	Sikkim
S-03-12	92.60	35.4	2.61	9	3.6	13.8	3.2	East	Sikkim
S-03-13	87.99	33.68	2.61	9.4	3.2	13	3	East	Sikkim
S-03-14	83.40	32.66	2.55	9.4	3.6	14.6	3.4	East	Sikkim
S-03-15	104.13	50.4	2.06	9.8	3.6	14.4	3.4	East	Sikkim
S-03-16	92	42.53	2.16	9.4	3.6	13.6	2.8	East	Sikkim
S-03-17	95.40	45.86	2.08	9.2	3.6	14.6	3	East	Sikkim
S-03-18	87.66	35	2.5	10.4	4.4	16.2	4	East	Sikkim
S-03-19	84	33.26	2.52	10	4.6	16.6	3.6	East	Sikkim
S-03-20	83.40	32.66	2.55	10.4	4.6	16.8	3.4	East	Sikkim
S-03-21	83.66	31.4	2.66	10.6	4.6	16.2	4	East	Sikkim
S-03-22	89.26	34.73	2.57	10	4.6	16.6	3.6	East	Sikkim
S-03-23	89.80	35.2	2.55	10.8	4.6	16.8	3.4	East	Sikkim
S-03-24	90.60	36.13	2.5	10.8	4.6	16.4	3.4	East	Sikkim
S-03-25	80.86	32.33	2.5	10.8	4.2	16.8	3.8	East	Sikkim
S-03-26	92.86	37.26	2.49	10.6	4.6	16.8	3.4	East	Sikkim
S-03-27	81.20	31.46	2.66	10.8	4.2	16.2	4.4	East	Sikkim
S-03-28	81.60	31.46	2.57	10.8	4.2	17.8	4.6	East	Sikkim
S-03-29	82.60	27.6	2.55	11	5	17.8	3.4	East	Sikkim

S-03-30	89.93	40.73	2.2	9.8	3.6	14.6	3.6	East	Sikkim
S-03-31	90.86	41.93	2.16	9.8	3.6	13.8	4	East	Sikkim
S-03-32	91.60	39.66	2.3	9	3.6	14.8	3	East	Sikkim
S-04-01	83.33	36.13	2.3	9.8	3.4	13.2	2.8	West	Sikkim
S-04-02	94.20	38.73	2.43	9.6	3.4	13.4	2.8	West	Sikkim
S-04-03	91.46	35.53	2.57	9.6	3.4	14.8	2.8	West	Sikkim
S-04-04	90.06	36.73	2.45	9.8	3.4	13.2	2.8	West	Sikkim
S-04-05	105.80	38.26	2.76	9.6	3.4	13.4	2.8	West	Sikkim
S-04-06	94.80	35.66	2.65	9.6	3.4	14.8	2.8	West	Sikkim
S-04-07	92.60	37	2.5	10.4	3.6	13.2	3.2	West	Sikkim
S-04-08	97.53	36.86	2.64	9.6	4	13.8	3.6	West	Sikkim
S-04-09	92.46	35.2	2.62	10	3.4	15	3.2	West	Sikkim
S-04-10	77.53	36.2	2.14	10.6	3.8	13.8	3.4	West	Sikkim
S-04-11	92.93	36.2	2.56	9.8	3.8	14.8	4.6	West	Sikkim
S-04-12	92.80	32.06	2.89	9.6	3.6	14.6	3.2	West	Sikkim
S-04-13	89	35.53	2.5	10.6	3.8	13.8	3.4	West	Sikkim
S-04-14	102.80	38.6	2.66	9.8	3.8	13.6	4.6	West	Sikkim
S-04-15	93.40	34.86	2.67	9.6	3.6	14.6	3.2	West	Sikkim

Visual Descriptor of Sikkim and Darjeeling mandarin

Plate 1.A. Tree growth habit: Spreading **B.** Shape of fruit base: Concave; Shape of fruit: Obloid (S-3-01/East Sikkim)

Plate 2.A. Tree growth habit: Erect; **B.** Shape of fruit base: Necked/Truncate; Shape of fruit: Obloid (D-01-07/Darjeeling)

Plate 3.A. Tree growth habit: Spreading; **B.** Shape of fruit base: Convex; Shape of fruit: Obloid (D-01-13/Darjeeling)

Plate 4.A. Tree growth habit: Spreading; **B.** Shape of fruit base: Truncate; Shape of fruit: Obloid (S-03-30/East Sikkim)

Plate 5.A. Tree growth habit: Erect; **B.** Shape of fruit base: Truncate; Shape of fruit: Obloid (S-03-24/East Sikkim)

Plate 6.A. Tree growth habit: Spreading; **B.** Shape of fruit base: Concave; Shape of fruit: Obloid (D-01-01/Darjeeling)

CHAPTER 5

Pollen morphology of *Citrus reticulata* Blanco of Darjeeling and Sikkim mandarin

5.1 INTRODUCTION

Citrus cultivars are derived from a variety of wild *Citrus* species (Taia et al. 2020). Some are pure wild-type choices, while others are crossbreeds between two or more ancestors (Taia et al. 2020). *Citrus* species are widely grown and valued for their fruit (AL-Anbari et al. 2015). *Citrus* has about 60 species, the majority of which are cultivated in the tropics and subtropics (AL-Anbari et al. 2015). However, *Citrus* species and allied genera are difficult to differentiate morphologically and geographically because there is a great deal of genetic variation in the species, as well as natural hybridization (Moore 2001).

Likewise, the mandarin group had many cultivars, which were mostly replicated by hybridization or mutation (Uzun and Turgut 2011). Mandarin is considered as a highly heterogeneous species among the three *Citrus* (Campos et al. 2005). Sexual hybridization among a large number of species and intra-specific hybrids have been attributed to the mandarin group's diversity. Furthermore, somatic mutation contributes to the rise in variety in this population in a different way (Coletta Filiho et al. 1998). The cross-pollinated nature of *C. reticulata*, and propagation through seed without nucellar section, traditionally followed in this region have produced considerable variation (Das et al. 2004a). Even within the same orchard, this substantial variance in phenotypic features such as plant types and fruit quality metrics can be seen (Das et al. 2003). The variation may also be caused because of the routine of planting trees from seeds of diverse and unknown origins (Dorji and Yapwattanaphun 2015). Based on the aforesaid reason, Dorji and Yapwattanaphun (2015) suggested Bhutanese mandarin comprise clones of multiple varieties. A study on the diversity of Himalayan *Citrus* using both morphological and Random Amplified Polymorphic DNA (RAPD) research indicated a wide variation in the north-eastern Himalayan region of India (Das et al. 2005).

As a result, the genus' taxonomy and systematics are complicated, and the exact number of natural species is unknown due to hybridization caused by sexual compatibility between *Citrus* and related genera, as well as within species within the genus (Gmitter and Hu 1990). In order to deal with this issue, morphological attributes are generally considered to ascertain the identification and diversity of *C. reticulata* (Dorji and Yapwattanaphun 2011a; 2011b). Besides the morphological assessment, the study of palynological character has been proved to be one of the pertinent taxonomic pieces of evidence for several plant groups (Kahraman et al. 2009; Asmat et al. 2011; Ceter et al. 2013). With robust extended research in the field of science, the application of palynological investigations in solving taxonomic difficulties is gradually gaining footing (Inyama et al. 2015). In the case of Rutaceae, several researchers looked at the pollen morphology of a few Rutaceous species and discovered their taxonomic importance at various taxonomic levels depending on the plant families (Erdtman 1952; Perveen and Qaiser 2005). However, there are no reports on pollen morphology of *C. reticulata* from Darjeeling and Sikkim Himalayas.

According to Davis and Heywood (1973), pollen characters are highly significant at the species and generic levels of taxa or among higher levels. The study of pollen morphology has been adopted a long time ago (Erdtman 1952) and is continuing to this day. Inyama et al (2015) differentiated among the members of *Citrus* while Taia (2004) showed the differences among the genera of tribe Trifolieae (Leguminosae) using pollen characters. Taia et al (2020) also studied pollen morphological variations among cultivated *Citrus* species and its related genera and revealed pollen size, shape, colpi length, apertures number and type, ora size, exine ornamentation, mesocolpium diameter are to be distinguishing characters of taxonomic value. Likewise, pollen morphology was used to search for a new character that could be helpful in the infrageneric classification of genus *Bomarea* (Sarwar et al. 2015). Sarwar et al (2015) further argue that morphological, palynological, and molecular data can be used to produce the most credible infrageneric classification.

Chapter 4 presents result that suggest Sikkim mandarin and Darjeeling mandarin to be morphologically varied due to the underlying genetic diversity. However, to make the result more credible this study aims to assess the relationship among mandarins grown in Sikkim and Darjeeling Himalaya using pollen morphological characters as suggested by Sarwar et al (2015).

5.2 MATERIALS AND METHODS

5.2.1 Study site

The collection sites were selected based on the results obtained in our investigation in Chapter 4 wherein accessions placed distinctly apart because of their varied quantitative characters were identified. The present investigation was carried out on mature trees of Sikkim and Darjeeling mandarin. Four uniform trees were selected from each orchard, from which mature anthers were taken from the uppermost flowers of the branches to obtain the mature pollen grains used in this study. The coordinates and altitude of the orchard were recorded using Global Positioning System (Garmin GPS – 12) (Fig. 20).

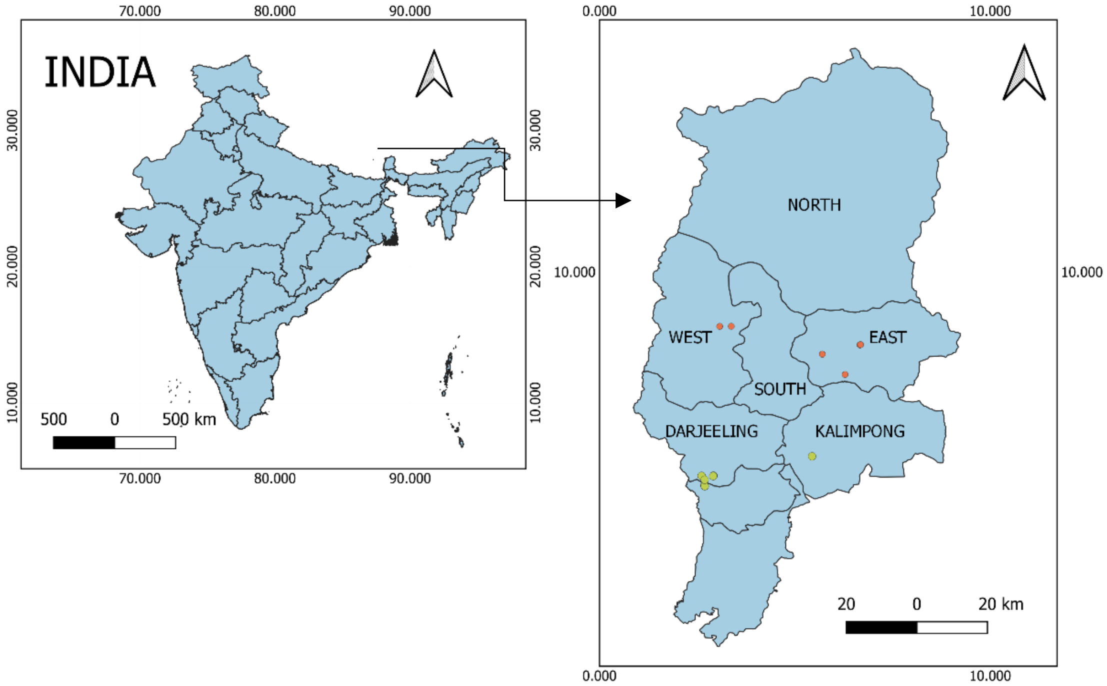


Fig. 20. Collection sites of pollen samples of *C. reticulata* from Darjeeling and Sikkim Himalayas

5.2.2 Preparation of pollen samples

The pollen grain samples so obtained were acetolyzed according to Erdtman's technique (Erdtman 1952). Pollen grains of the acetolyzed portion were dehydrated in ethanol series placed onto coverslips, left for ethanol evaporation then attached to copper stubs by double-sided tape, coated with 30 nm gold using fine coat ion sputter JEOL JFC 1100E, examined and photographed using Zeiss scanning electron microscope. The terminology used in the present study is according to Faegri (1956) and Erdtman (1952).

5.2.3 Statistical analysis

For all the studied accessions, the mean values of the pollen characters were separated and calculated then compared using the least significant difference (LSD) test at 0.05 level of probability (Snedecor and Cochran 1990). The statistical analysis was performed using SPSS, IBM. Principal Component Analysis was performed using R ver. 4.1.2.

6.3 Results

6.3.1 Multivariate analysis of pollen descriptors

The results obtained from the fourteen studied accessions are summarized in Table 8 and illustrated in Fig. 22-35. The pollen grains of all taxa were monads, radially symmetrical, isopolar, and were different in size. The pollen shape varied from prolate (P/E 1.38) to sub-prolate (1.24) (Table 8). The mean polar length ranged from 29.37 µm (D-01-14) to 35.07 µm (S-03-16). The aperture type was observed to be tetracolporate whereas exine ornamentation was observed to be microreticulate (Table 8). The ecto-aperture colpi in all the studied accessions were long, narrow, and equally spaced around the equator. The mean colpi length varied within the studied taxa from a minimum of 25.8 µm (D-01-09) to 30.47 µm (D-01-05).

A one-way ANOVA performed on the quantitative characters of pollen showed high significant variation among the pollens of accessions collected from several locations (Table 7). Principal component analysis revealed the first two principal components to contribute to the majority of the total cumulative variance. PC1 and PC2 contributed to a total of 98.3% variance (Fig. 21). While the accessions of Darjeeling were concentrated towards the positive quadrant of PC2 and negative quadrant of PC1 and the accessions were characterized by the equatorial diameter of the pollens, the accessions from East Sikkim were largely concentrated towards the negative quadrant of PC2 and positive quadrant of PC1 (Fig. 21). The accessions were characterized by the pollen length, colpi length, and ratio of pollen length to equatorial diameter (Fig. 21).

Table 7: Result of ANOVA testing for differences in pollen traits of selected accessions

Sl. No.	**Traits**	DF	F	P-value	Significance
1	Mean polar Length	13	6.96	0.0001	***
2	Mean equatorial diameter	13	4.28	0.0001	***
3	Colpi Length	13	7.25	0.0001	***
4	Polar Length/Equatorial Diameter	13	4.87	0.0001	***

*(ns, non-significant, $p > 0.05$; ***$p < 0.0001$)*

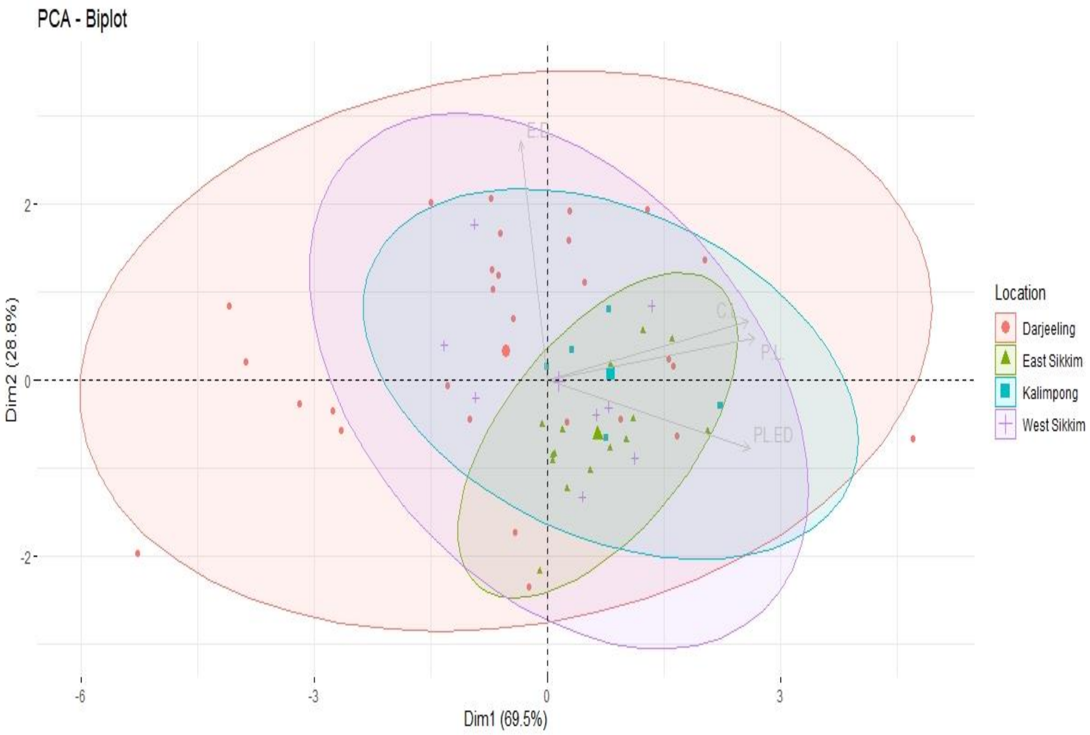


Fig. 21. Principal Component analysis depicting the contribution of 4 quantitative characters for variation among the selected mandarin accessions of *C. reticulata*. The two components of principal component analysis (Dim 1 and Dim 2) are plotted on the axes.

Table 8: Pollen morphological characters of the studied Sikkim and Darjeeling mandarin (*C. reticulata*)

Sl. No.	**Characters →** **Accessions ↓**	**Location**	**P.L. (µm)**	**E.D. (µm)**	**PL/ ED**	**C.L.**	**Pol. Sh.**	**Ap.**	**Ex. Or.**
1	D-01-07	Darjeeling	33.67	24.38	1.38	28.4	Prolate	Tetracolporate	microreticulate
2	D-01-08	Darjeeling	33.82	24.33	1.39	28.7	Prolate	Tetracolporate	microreticulate
3	D-01-09	Darjeeling	31.06	22.98	1.35	27.3	Prolate	Tetracolporate	microreticulate
4	D-01-13	Darjeeling	33.77	23.06	1.46	28.4	Prolate	Tetracolporate	microreticulate
5	D-01-14	Darjeeling	29.37	23.59	1.24	25.1	Sub-prolate	Tetracolporate	microreticulate
6	D-01-15	Darjeeling	34.26	24.11	1.42	29.3	Prolate	Tetracolporate	microreticulate
7	D-01-05	Darjeeling	35.67	23.07	1.54	30.4	Prolate	Tetracolporate	microreticulate
8	S-03-16	East Sikkim	35.07	23.21	1.51	30.6	Prolate	Tetracolporate	microreticulate
9	S-04-10	East Sikkim	33.53	22.74	1.47	27.9	Prolate	Tetracolporate	microreticulate
10	S-04-14	East Sikkim	34.71	23.28	1.49	29.1	Prolate	Tetracolporate	microreticulate
11	S-04-11	East Sikkim	33.31	23.24	1.43	28.2	Prolate	Tetracolporate	microreticulate
12	S-04-08	West Sikkim	33.11	23.20	1.42	28.5	Prolate	Tetracolporate	microreticulate
13	S-04-02	West Sikkim	34.99	23.24	1.50	29.6	Prolate	Tetracolporate	microreticulate
14	S-02-05	Kalimpong	34.03	23.47	1.44	29.2	Prolate	Tetracolporate	microreticulate

P.L.: Polar length in µm; E.D.: Equatorial Diameter in µm; P/E: Ratio between Polar length/Equatorial diameter; Pol. Sh.: Pollen Shape; Ap.: Aperture number and type; Ex. Or.: Exine ornamentation; C.L.: Colpi length in

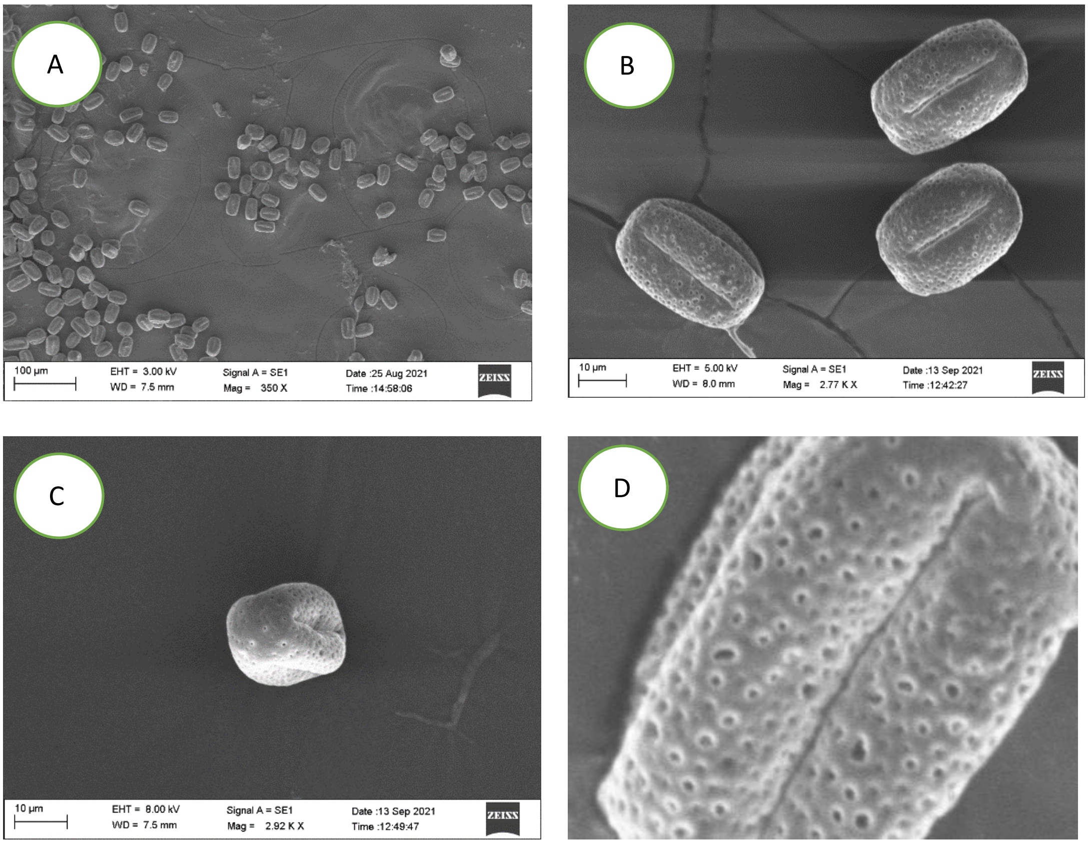
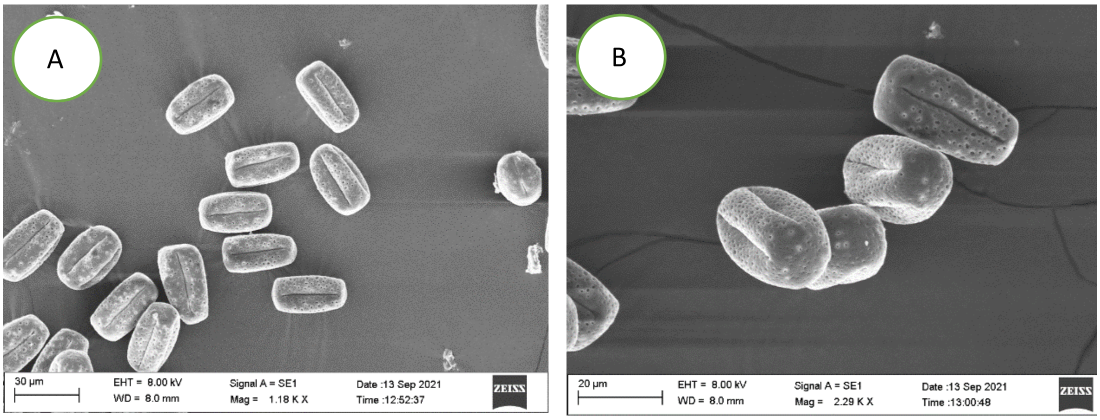


Fig. 22. SEM **(A-D)** photomicrographs of D-01-05 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation



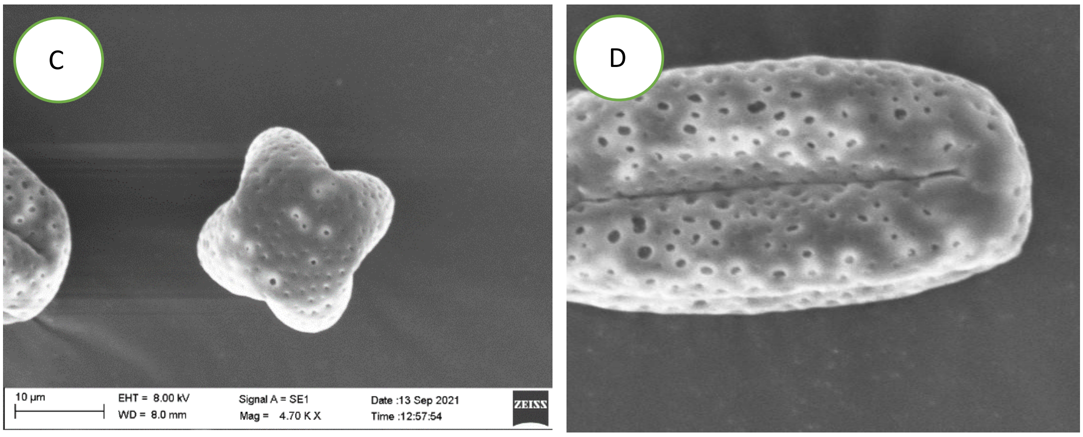


Fig. 23. SEM **(A-D)** photomicrographs of S-03-16 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation

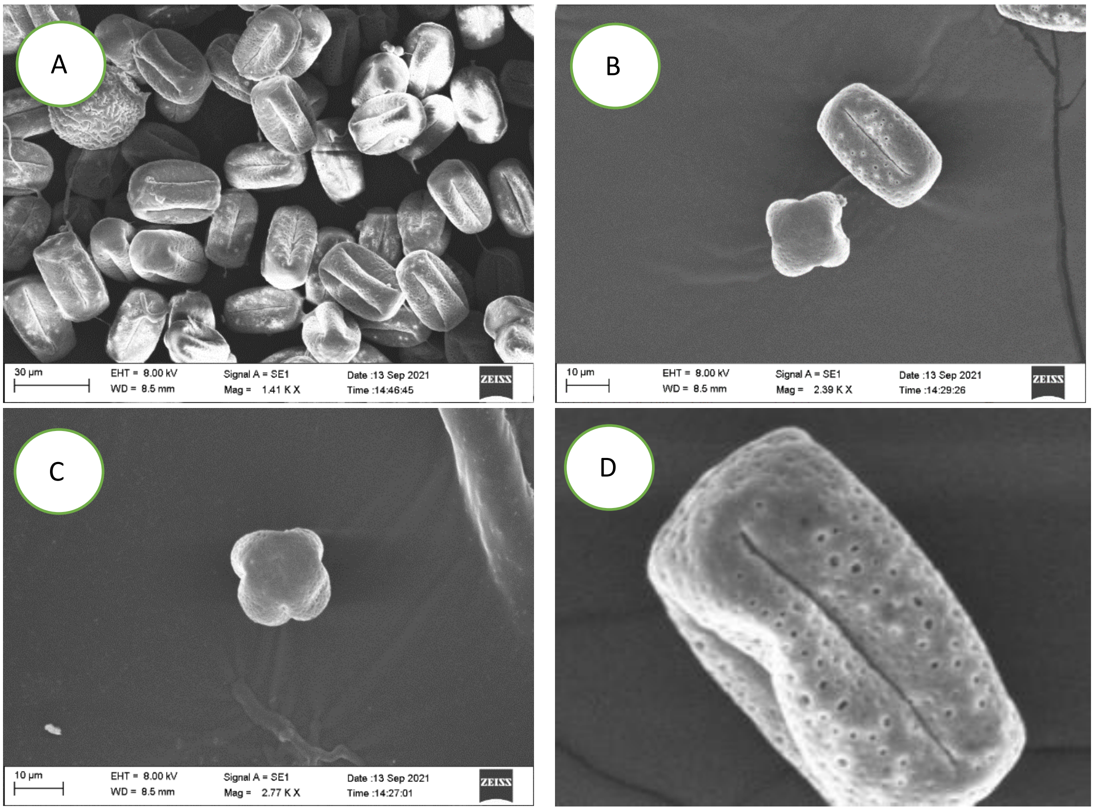


Fig. 24. SEM **(A-D)** photomicrographs of S-04-10 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation

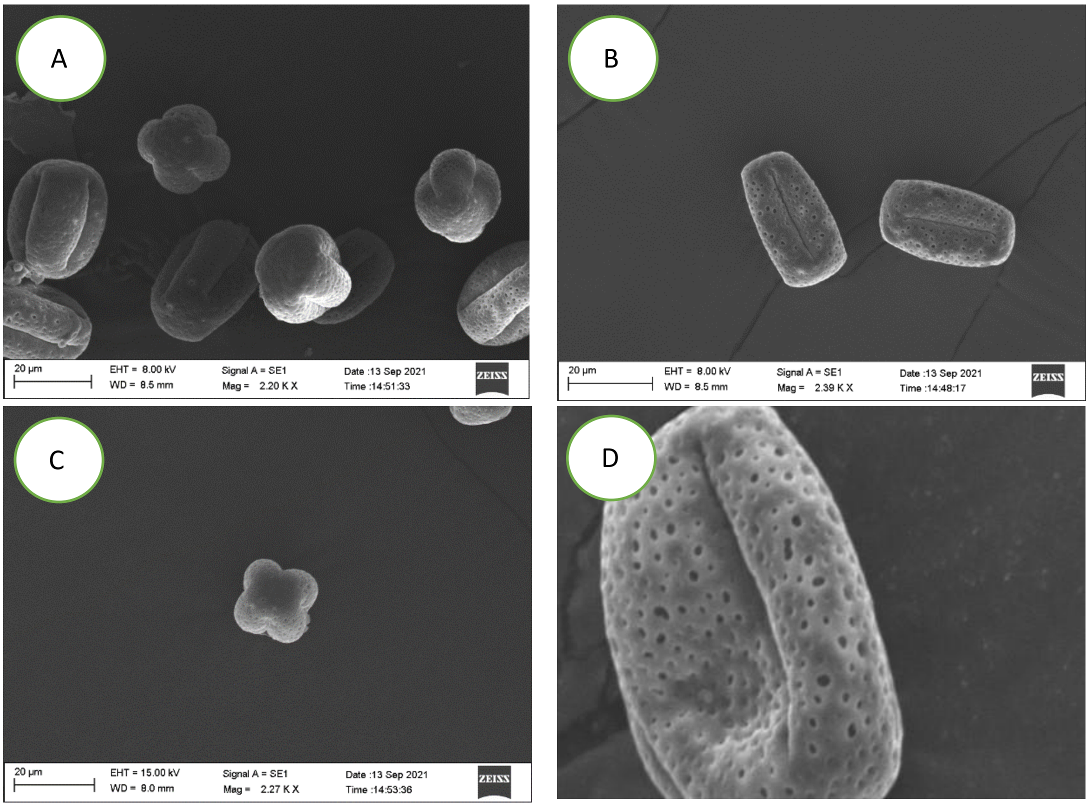


Fig. 25. SEM **(A-D)** photomicrographs of S-04-14 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation

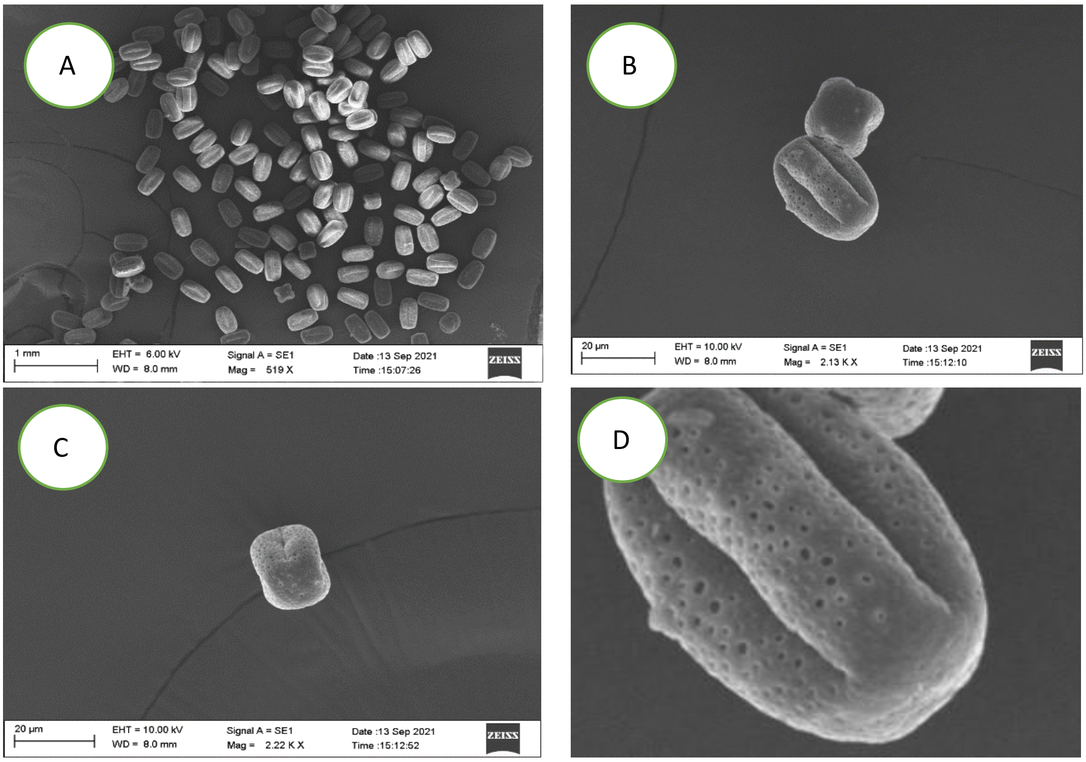


Fig. 26. SEM **(A-D)** photomicrographs of S-04-11 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation

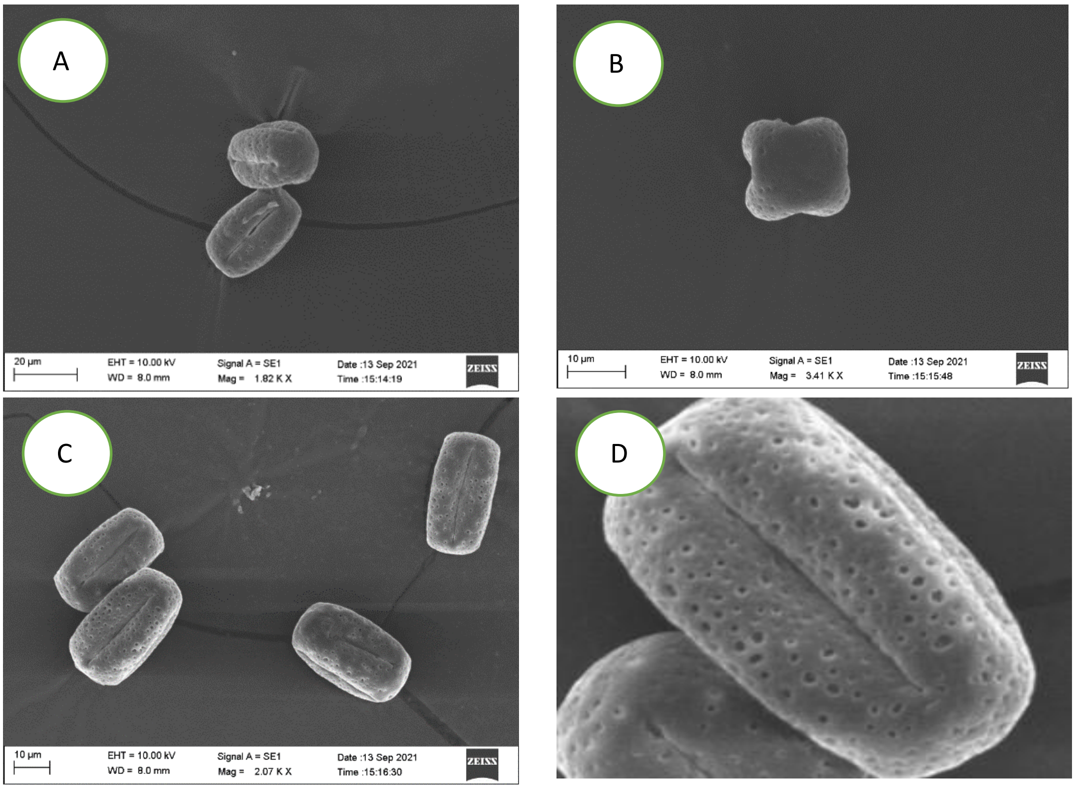


Fig. 27. SEM **(A-D)** photomicrographs of D-01-07 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation

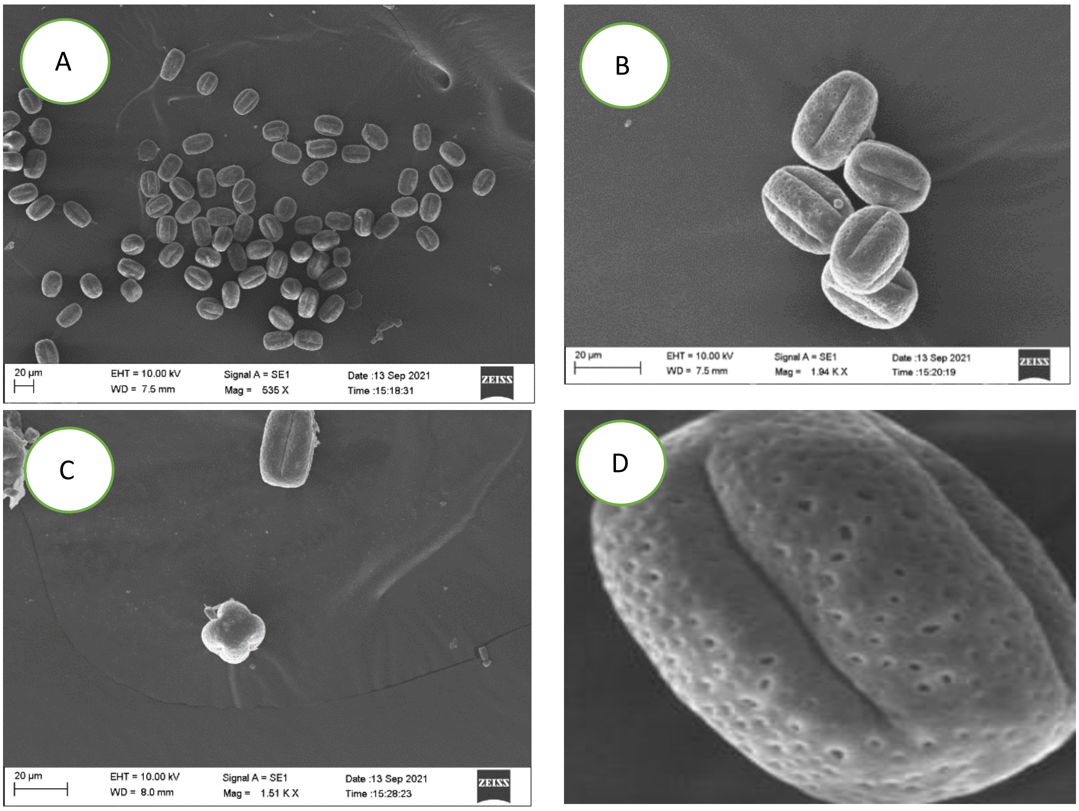


Fig. 28. SEM **(A-D)** photomicrographs of D-01-08 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation

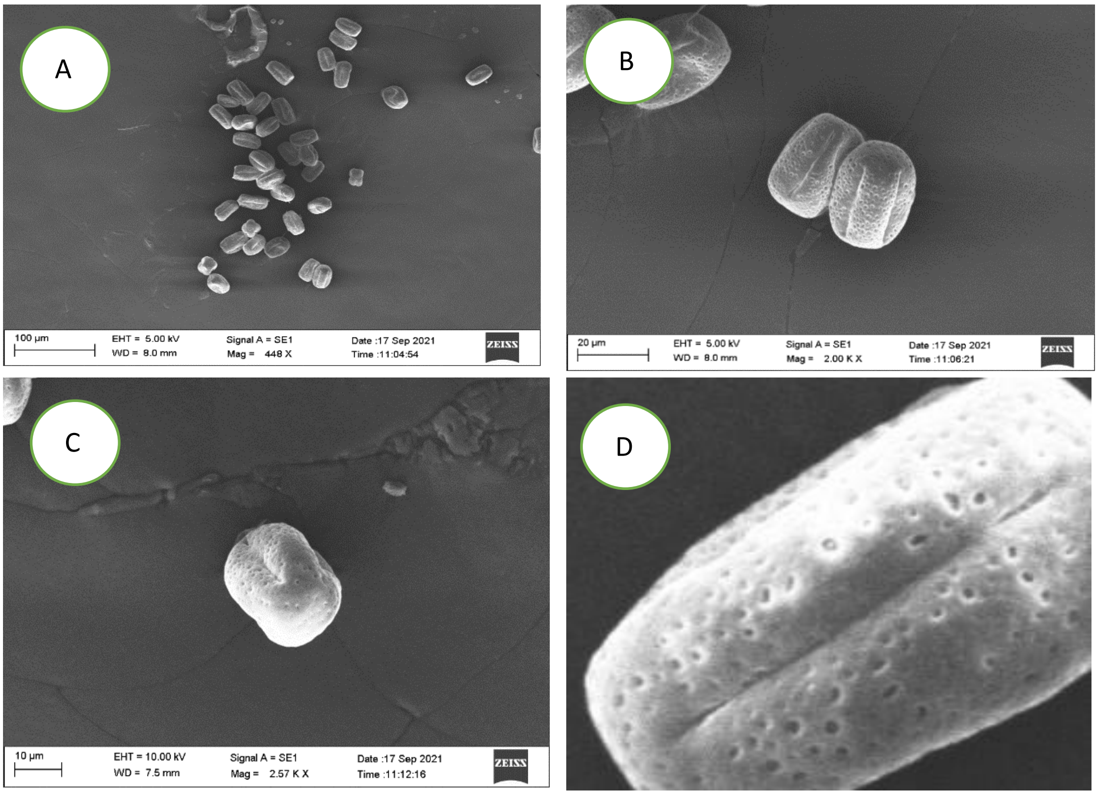


Fig. 29. SEM **(A-D)** photomicrographs of D-01-09 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation

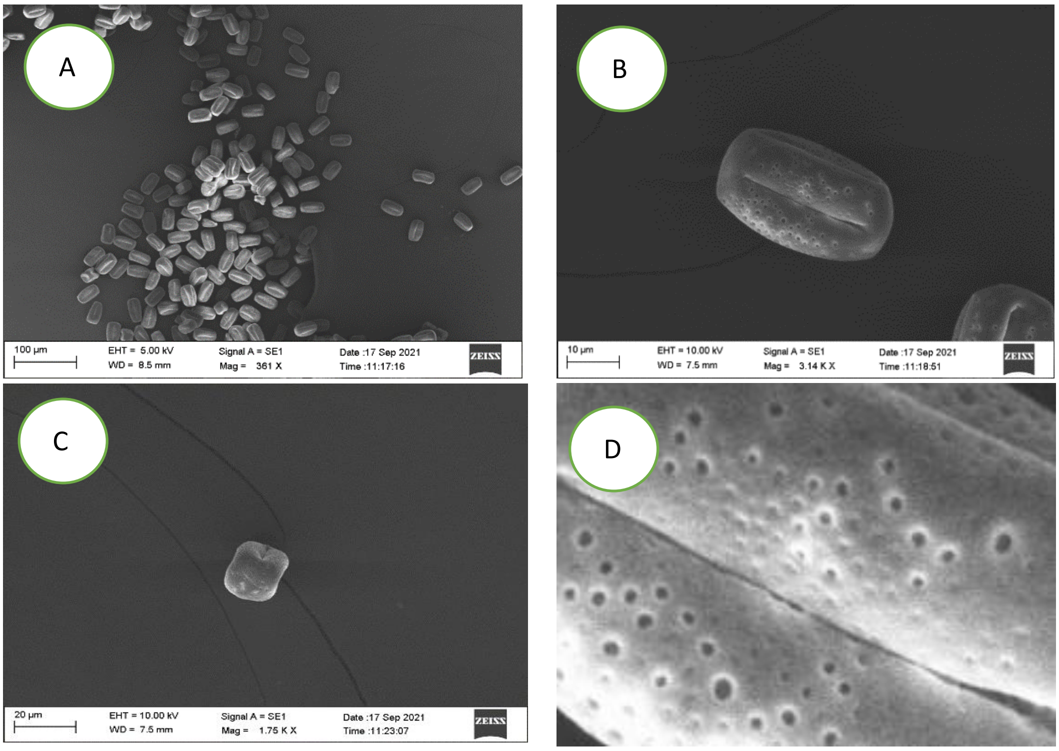


Fig. 30. SEM **(A-D)** photomicrographs of D-01-03 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation

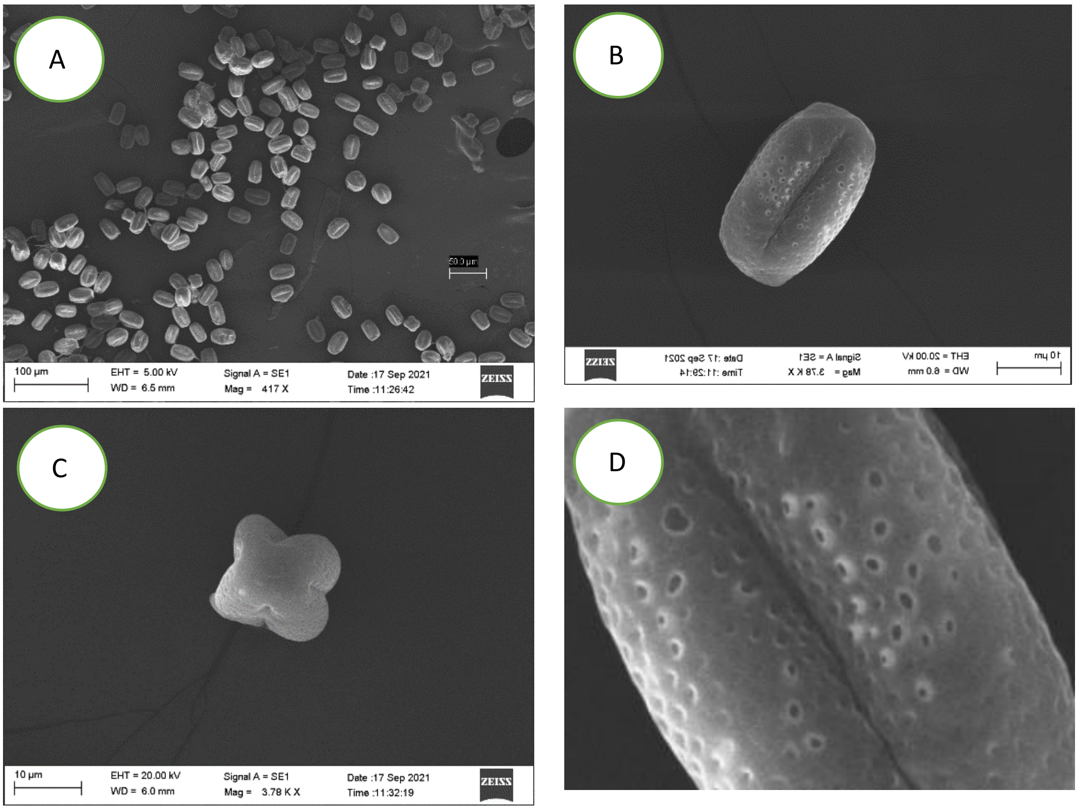


Fig. 31. SEM **(A-D)** photomicrographs of D-01-14 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation

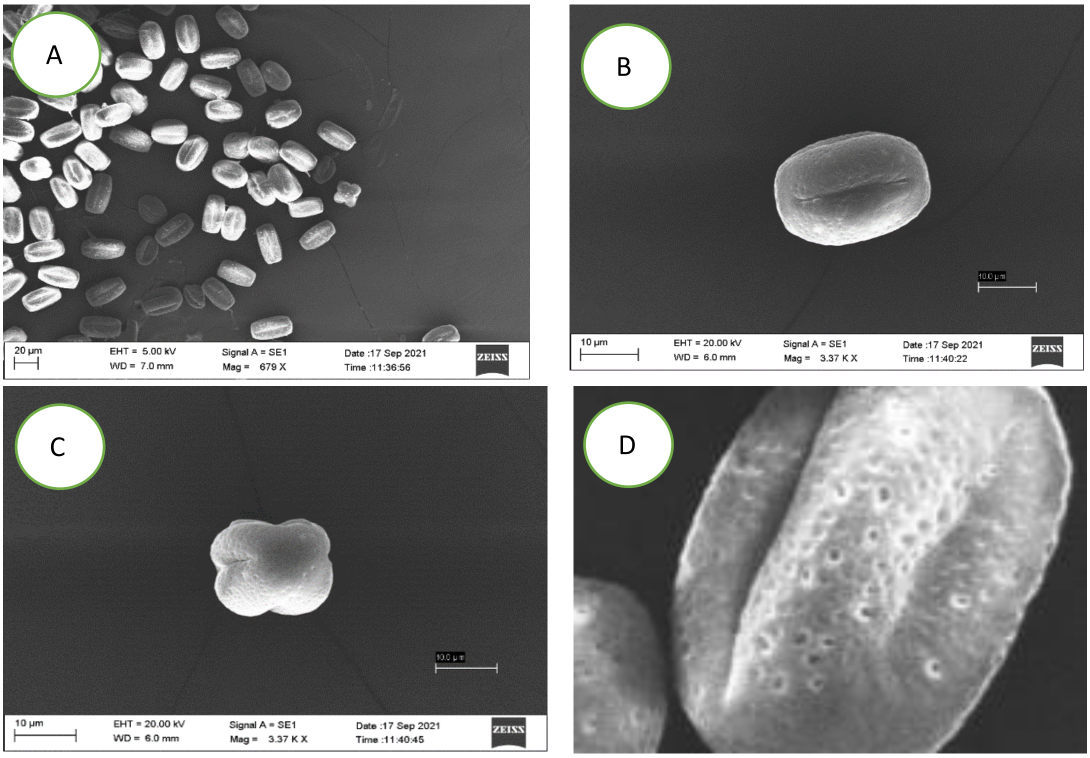


Fig. 32. SEM **(A-D)** photomicrographs of D-01-15 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation

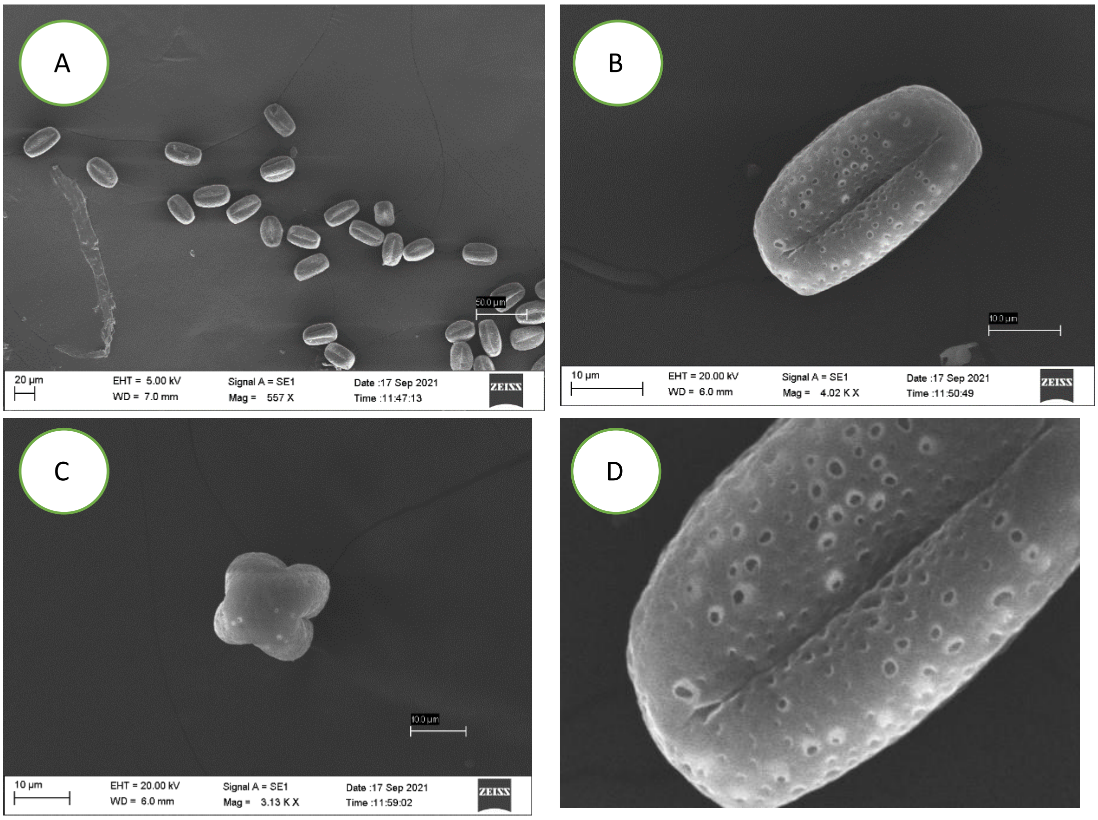


Fig. 33. SEM (**A-D**) photomicrographs of S-04-08 pollen grains; **A**. cluster of pollen grains **B**. pollens in equatorial view **C**. Single pollen in polar view **D**. Exine ornamentation

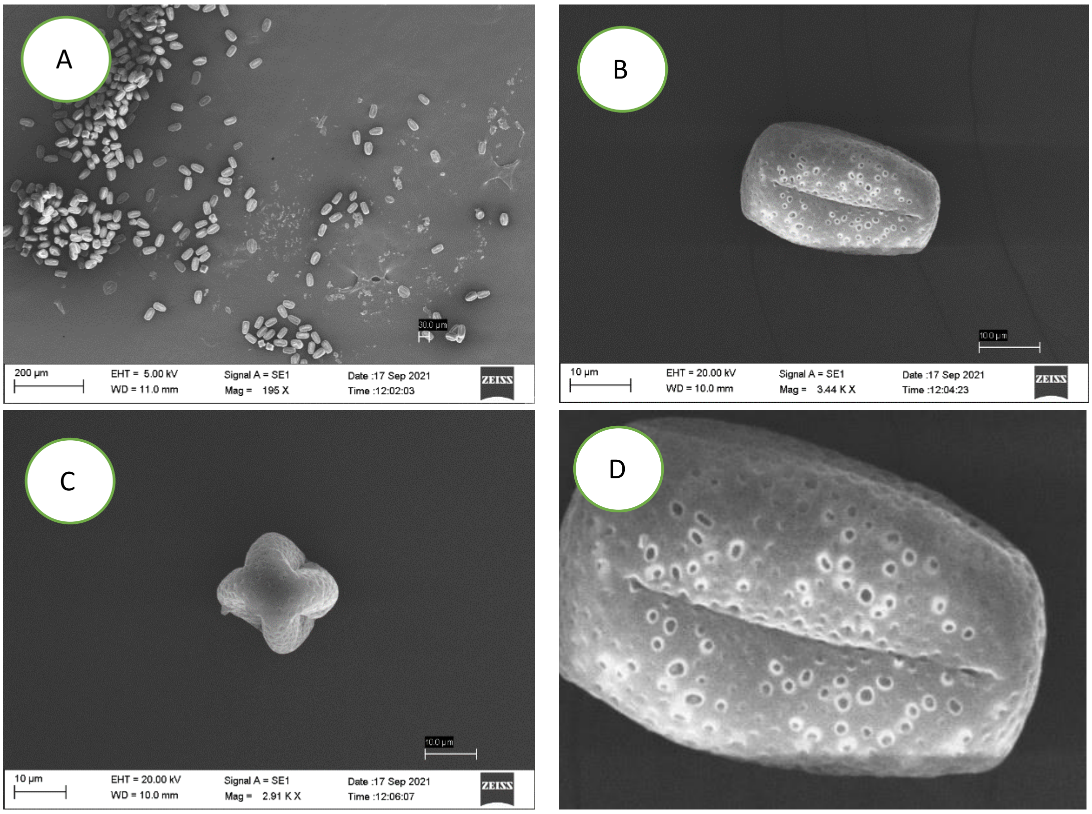


Fig. 34. SEM **(A-D)** photomicrographs of S-04-02 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation

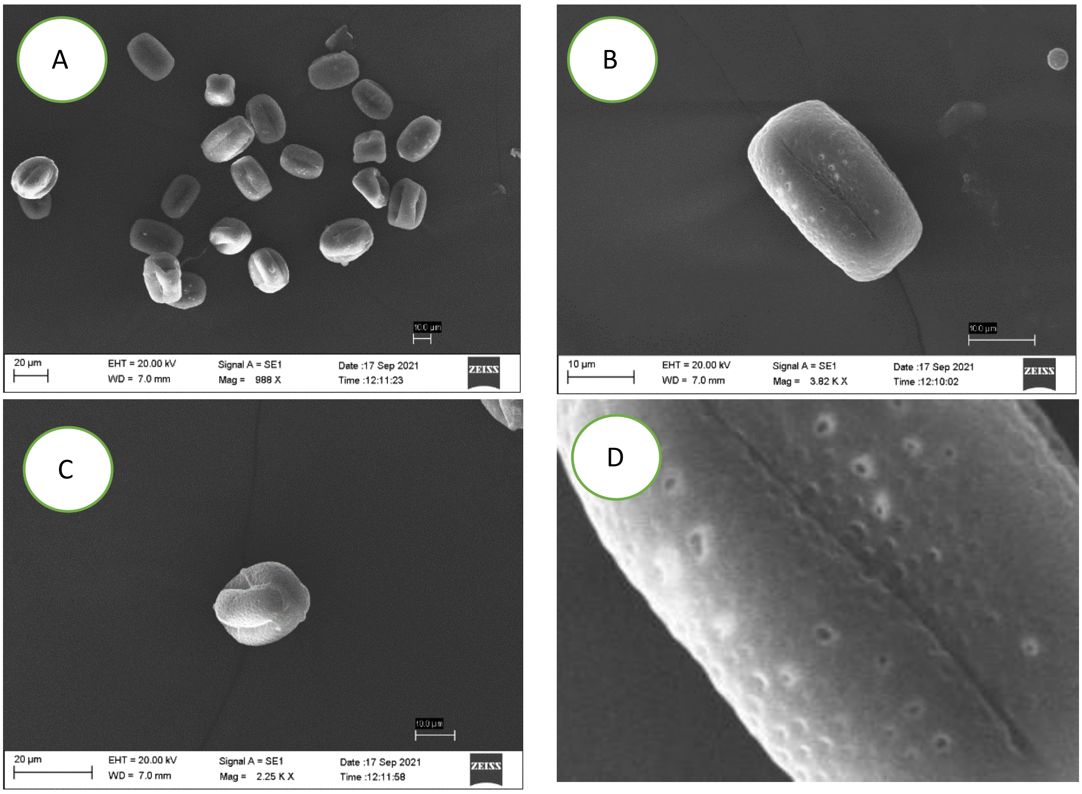


Fig. 35. SEM **(A-D)** photomicrographs of S-02-05 pollen grains; **A.** cluster of pollen grains **B.** pollens in equatorial view **C.** Single pollen in polar view **D.** Exine ornamentation

5.4 Discussion

The pollen morphological characters and ultrastructure have been used to identify and distinguish between species and cultivars of fruit trees (Asma 2008; Gilani et al. 2010; Nikolić and Milatović 2016). Determining pollen morphology and comparing the differences among species and cultivars improves our understanding of reproductive biology (Asma 2008). *C. reticulata* is a commercially desirable variety of the mandarin group and the most important commercial fruit of Sikkim (Lachungpa 2004; Pradhan and Devy 2018). However, there are no reports on pollen morphology of *C. reticulata* of Darjeeling and Sikkim Himalayas.

According to our results, pollen characters play a pivotal role in bringing about variations in mandarins. In **Chapter 4** we placed evidence that suggested mandarins have two ecotypes– Sikkim and Darjeeling mandarin. Additionally in the present study, palynological investigations indicated that variations in pollen morphological characters were of taxonomic significance. In particular, the four quantitative pollen characters of the studied accessions were significantly different from each other. On the contrary, Taia (2020) placed the ratio of polar length and equatorial diameter (P/E) to be of less taxonomic value. Meanwhile, equatorial diameter was the most contributing trait in accessions of Darjeeling while mean polar length and mean equatorial diameter was the pollen characters that were highly correlated and contributing trait in accessions of Sikkim mandarin. Similarly, polar length and equatorial diameter were considered to be taxonomically significant among the closely related taxa of *Citrus, Fortunella margarita*, *Citrofortunella floridana*, and *Poncirus* (Taia 2020). Although AL-Anbari et al (2015) found pollen shape and type of exine sculpturing to be useful characteristics for classification and identification there was no credible contribution of pollen shape in the classification of mandarin accessions of the present investigation. Inyama et al (2015) reported the pollen shape of *C. reticulata* of Owarri to be circular-elliptical while Taia (2020) reported the pollen shape to prolate-spheroidal. However, according to our analysis, the pollen shapes of our accessions were observed to be prolate or sub-prolate. The reason for such variability could be because mandarin is the most diverse group of *Citrus* species (Rahman et al. 1994; Koehler et al. 2003; Yamamoto et al. 2013) and consists of numerous intergeneric and interspecific hybrids (Abnekar et al. 2004; Warburton et al. 2005).

The findings of the present study on the size of pollen are in agreement with the previous study (Taia et al. 2020). Pollen grains have been classified into groups according to their sizes by Erdtman (1952) as perminuta (diameter less than 10 µm), minuta (diameter 10-25 µm), media (diameter 25-50 µm), magna (diameter 50-100 µm), permagana (diameter 100-200 µm) and giganta (diameter greater than 200 µm).

Based on this classification, the pollen grains of the studied accessions belong to group media (diameter 25-50 µm). The pollen grain size reported for this family supports the fact that flowers of *C. reticulata* are pollinated by honeybees where the wind is not a major factor (Mcgregor 1976).

Apertures and sculpturing of the exine wall make pollen grain a very recognisable object that can be used to identify genera or even a species (Scheel 1996). The studied accessions showed similar aperture and exine ornamentation. AL-Anbari (2015) suggested hybrid taxa to possess different types of apertures in the same plant while the original species mostly have one type. This confirms the assertion that *C. reticulata* is a true “fundamental” species from where other hybrids were derived (Barret and Rhodes 1976; Wu et al. 2018) and also indicates mandarin of Sikkim and Darjeeling Himalayas to be the original species of the region. Moreover, the north-eastern region of India is reported to be the centre of origin and rich in diversity of *Citrus* species (Hynniewta et al. 2014; Hazarika 2012; Wu et al. 2018).

The significant variation observed in the morphological characters of pollen and its contribution in placing Darjeeling accessions and Sikkim accessions in different quadrants on PCA adds on to the credibility of our findings in **Chapter 4.** According to AL-Anbari (2015), pollen analysis along with morphological and molecular studies should be considered to understand relationships, taxonomy, and variability in *Citrus*. Similar to the assessment of morphological characters in **Chapter 4** that determined mandarin accessions to be genetically differing resulting in morphological diversity, pollen characters contributed similarly in differing Sikkim and Darjeeling accessions.

CHAPTER 6

Pollination requirements of Darjeeling and Sikkim mandarin (*Citrus reticulata* Blanco) through ages of its domestication in the eastern Himalayas, India

6.1 INTRODUCTION

Pollinators are critical for effective reproduction where one-third of the world's food crops are dependent on animal pollination (McGregor 1976; Klein et al. 2007). Pollination benefits 70 percent of 1330 tropical crops (Roubik 1995) and 85 percent of 254 crops in Europe by improving their fruit and seed quality and enhancing the yield (Williams 1994). Similarly, many plantation crops, such as cardamom, coffee, and vegetable crops, rely on pollination as well (Partap 1999; Chandel et al. 2004; Sinu and Shivanna 2007a; 2007b). Studies suggest that the increased abundance of bumblebees and bees in the plantation increased the production of *Amomum subulatum* (Verma 1987, Gaira et al. 2016). *Coffea arabica* is dependent on wind and insect pollination which pronounced the productivity of coffee by 50 % (Krishnan et al. 2012). Similarly, experimentally multiplying the insect pollinators in apple orchards augmented the fruit set and yield (Stern et al. 2001; Ladurner et al. 2004).

Citrus, unlike other fruit crops, has a wide range of pollination requirements, which has resulted in inconsistent findings on its pollination response. *Citrus* blooms range varies from self-fertile to completely self-sterile, requiring pollen from other suitable varieties to be introduced to such self-sterile blossoms in order to increase output (McGregor 1976). In others, the transfer of pollen from flower to flower within the cultivar or species is beneficial while in some, flowers' own pollen is adequate for maximum fruit production (McGregor 1976). *Citrus* has been reported to be a crop that requires little or no insect pollination (McGregor 1976). Domestication has resulted in the production of hybrids over time, with the practice of grafting (Nicolosi et al. 2005). Domestication also causes crop species to alter in comparison to their wild ancestors (Colunga-GarciaMarin et al. 1996; Casas et al. 1999; 2007). ‘Interdonato’ lemon was reported to be a citron × lemon hybrid (Deng et al. 1995). Further, pollination by other *Citrus* hybrids forms citron hybrids when propagated by seeds (Nicolosi et al. 2005). As a result, despite its sexual reproduction, the variations do not remain completely static (Webber and Batchelor 1943), and the possibility that pollination requirements have evolved over time cannot be neglected. Lemon trees that were not pollinated by bees produced only one-fourth as much fruit as trees that were pollinated by bees (Glukhov 1955). Honeybees enhanced the fruit set of oranges by 31%, increased the fruit weight by 22%, increased the amount of juice by 33%, and increased the number of seeds by 36%. (Wafa and Ibrahim 1960). Bees, on the other hand, had little effect on the production of Valencia (Francke et al. 1969), but cross-pollination with Pearl tangelo pollen resulted in an increase in fruit set and seed number.

Among the *Citrus* species, pollination problem has been reported to be severe in mandarin and mandarin-hybrid complex (Mcgregor 1976). Some mandarins exhibit some degree of apomixis (Iglesias et al. 1996), whereas others are self-incompatible, sterile, parthenocarpic, and produce defective pollen (Baldwin 1993; Davis and Albrigo 1994). When Clementine tangerine was cross-pollinated by bees with Dancy, Temple, Duncan or other seedy cultivars, its yield rose (Oppenheimer 1948). Lee, Page, Nova and Robinson (Hearn et al. 1969) along with Orlando, Minneola and Osceola were found to be self-incompatible (Krezdorn 1972), whereas Hyuganatsu mandarin was self-sterile but cross-fertile (Miwa 1951).

Citrus and mandarin cultivars have such a wide range of pollination requirements that determining the breeding system and pollination biology of mandarin cultivars is difficult. The declining productivity of Sikkim mandarin and Darjeeling mandarin in the eastern Himalaya is a matter of concern, as it has long been linked to pest and disease outbreaks, a lack of planting material, and poor management (Biswas et al. 2016; Gurung et al. 2017). Considering the dynamic nature of mandarins concerning its breeding system, the present study addresses the following questions: Is mandarin self-compatible? (ii) What is the level of dependency of mandarin on pollination? (iii) What is the reproduction success of mandarins? (iv) Can a better understanding of the breeding system resolve the issue of crop yield?

6.2 Materials and Methods

6.2.1 Study site

The first study site was an orchard in "Dzongu" in the northern district of the state of Sikkim, India (27° 28' 25.4" N, 88° 30' 36.1" E, 1151 m a.s.l.) during the peak flowering season in March 2018 and 2019.

The study was conducted in an orchard in "Dzongu" (Orchard A) in the northern district of the state of Sikkim, India (27° 28.423' N, 88° 30.602' E, 1151 m a.s.l.) (Fig. 36) during the peak flowering season in March 2018 and 2019. The orchard expands to an area of about 1000 m^2 and contains plantations of a single variety of *C. reticulata* commonly known as Sikkim mandarin in the eastern Himalaya. The temperature of the study site varies between a minimum of 14.70 °C and a maximum of 23.82 °C and rainfall of 211.16 mm annually. The study site comprises subtropical types of semi-natural habitat comprising of species such as *Alnus nepalensis*, *Albizia* spp., *Macaranga* spp., *Juglans regia*, *Myriocarpa* spp. in abundance.

The second study site was an orchard in “Nagri Tea Estate” (Orchard B) in Darjeeling district the state of West Bengal, India (26° 55’ 36” N, 88° 11’ 15” E, 1351 a.s.l.) (Fig. 36) during the peak flowering season in March 2018 and 2019. The orchard expands to an area of about 800 m^2 and contains plantations of a single variety of *C. reticulata* commonly known as Darjeeling mandarin in the eastern Himalaya. The temperature of the study site varies between a minimum of 12.10° C and a maximum of 25.20° C and rainfall of 205.12 mm annually. The study site comprises subtropical types of semi-natural habitat comprising of species such as *Schima wallichii*, *Choerospondias axillaris*, *Alnus nepalensis*, *Prunus* sp., *Terminalia elliptica*, and vast plantation of *Camellia sinensis* var. sinensis.

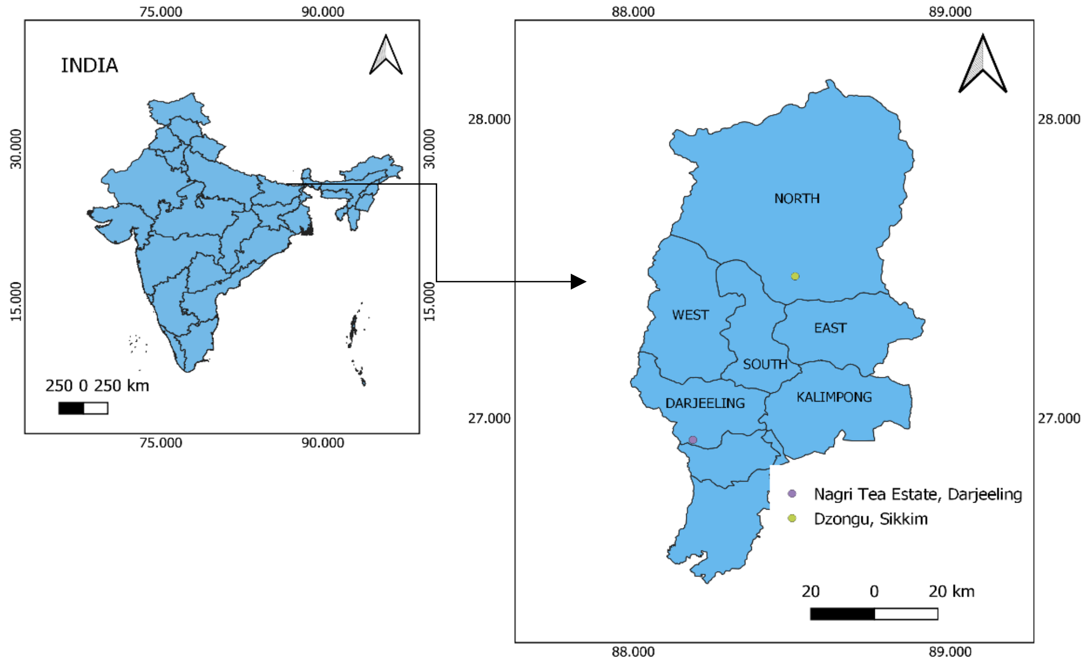


Fig. 36. Study site in north Sikkim and Darjeeling, the eastern Himalaya

6.2.2 Field data collection

6.2.2.1 Pollination activity

Preliminary observations were made, and an observation period of the day was decided. Accordingly, seven healthy trees of *C. reticulata* that were grown in separate rows were randomly selected, and a plot was chosen containing 30 open flowers (Shivanna and Tandon 2014). A visitor is considered a pollinator only if it comes in contact with the anther and stigma while foraging. The frequency of the visits by the pollinators inside the plot was noted at an hourly interval starting from 0600 - 1800 h for seven days (a total of 168 h in both the years) spanning from 6th March – 12th March 2018 and 10th March – 16th March 2019 in Orchard A. Similarly, the study was conducted on 14th March – 20th March 2018 and 16th March – 22nd March 2019 in Orchard B during the peak flowering season. The seven healthy trees selected for the study were observed in succession for seven days.

6.2.2.2 Pollination treatments

The breeding system of mandarin orange and pollinator dependency was estimated by performing hand-pollination experiments. Five sets of flower buds were selected randomly from the same seven healthy trees and were bagged before anthesis and anther dehiscence. All five sets were tagged with different coloured ribbons to differentiate between the experiments. The first set of flower buds (N=100) was bagged without emasculation to test autogamy. On the day of anthesis, the bags were opened, and the flowers were pollinated with pollen from freshly dehisced anther of the same flower. The second set of flower buds (N=100) was bagged after emasculation to test apomixis. To test for geitonogamy, the third set (N=100) was bagged after emasculation. On the day of anthesis, the bags were opened, and the emasculated flowers were pollinated with pollen from freshly dehisced anther of another flower of the same plant and rebagged. To test cross-pollination, the fourth set of flower buds (N=100) were bagged after emasculation. On the day of anthesis, the bags were opened, and the emasculated flowers were pollinated with pollen from freshly dehisced anther of the flower of another plant and rebagged. The fifth set (N=100) was left untreated to test open pollination (Shivanna and Tandon 2014). In order to test the pollination efficiency (PE), another set of flowers was bagged in which a pollinator was allowed to visit only once after which the flowers were bagged again (Shivanna and Tandon 2014). All the flowers were kept under observation and the matured fruits were collected on 2nd November 2018 and 12th November 2019 in "Dzongu" and 7th November 2018 and 8th November 2019 in "Nagri Tea Estate". The results of the breeding experiment performed were estimated by noting the % of fruit set in each set of flowers during the fruiting season.

6.2.2.3 Breeding System

The breeding system of the species was analysed using the index of self-incompatibility (ISI) (Zapata and Arroyo 1978) which is the ratio of fruit set in self-pollinated flowers to fruit set in cross-pollinated flowers. The species with ISI 1 or >1 is considered fully self-compatible, > 0.2 but < 1 is considered as partially self-compatible and < 0.2 or 0 as fully self-incompatible (Zapata and Arroyo 1978)

6.2.2.4 Pollinator dependency

Pollinator dependency (PD) was estimated by subtracting the % fruit set through autogamy from % fruit set by open pollination (Tur et al. 2013). The PD index ranges from 0 (where the plants are not dependent on pollinators) to 100 (where the plants are completely dependent on pollinators).

6.2.2.5 Pollination efficiency

Pollination efficiency was investigated based on seeds formed per visit (Shivanna and Tandon 2014) and was evaluated using Spear's pollination index = $\frac{Pi-Z}{U-Z}$ where Pi is the average number of seeds in the fruit after only one visit by the pollinator i, z is the average number of seeds in the fruit that did not receive any visit by the pollinator and U is the average number of seeds in the fruits that received unrestricted visit (Spears 1983). The index ranges from 0 (where the pollinator does not contribute anything) to 1 (where the production of fruit or seed by a pollinator is equal to the flowers left for open pollination).

6.2.2.6 Pollen limitation

The pollen limitation was estimated as the ratio of fruit set in cross-pollinated flowers to fruit set in open-pollinated flowers (Larson and Barrett 2000). The scale ranged from 0 - 100 where 0 indicated no pollen limitation to 100 indicating pollen limitation.

6.2.2.7 Pollen fertility

Pollen fertility was estimated by in-vitro germination of pollen by sitting drop culture method (Shivanna and Tandon 2014). A thin layer of pollens was spread on a dry microscope slide at 0, 3, 6, 9, 24 h after its anthesis and kept in a humidity chamber for prehydration for 30-60 minutes. A total of four replicates of pollens collected from four different trees were cultured at each pre-set time. The prehydrated pollens were dispersed in a drop (20-30 µl) of standard germination medium taken on a microscope slide. Standard medium was prepared by mixing 10% sucrose and 100 mg/l Boric acid. The pollen cultures were then kept in the humidity chamber for 2 h before it was scored to calculate the percent germination.

6.2.3 Quantitative and Physico-chemical analysis

The fruits from different sets of pollination treatments were collected and the morphological characters (IPGRI 1999) such as height, diameter, weight, number of seeds per fruit were determined. The acidity of the juice was estimated by acid-base titration while total soluble solids (TSS) were estimated by a hand refractometer (ERMA, Japan).

6.2.4 Quantitative Phytochemical analysis

6.2.4.1 Preparation of juice samples

Fresh fruits of mandarin from different pollination treatments were obtained from both the study sites in November 2018 and 2019. The fruits were cut into half and hand-squeezed to extract the juice. The juice was then passed through the strained to remove pulp and seeds. The processed juice was then collected and stored at -20°C until further analysis.

6.2.4.2 Determination of total phenolic content

The total phenolic content of mandarin was measured following Velioglu et al (1998) with minor modification. The juice sample (1 ml) was mixed with 5 ml of 10-fold diluted Folin Ciocalteu reagent and left at 22°C for 5 minutes. 0.75 ml of Na_2CO_3 (60g/l) solution was further added and left at 22°C for 90 minutes. Thereafter, the absorbance was estimated at 725 nm using a UV-visible spectrophotometer. Gallic acid was used to create the standard curve, with concentrations ranging from 10-100 µg/ml. Results were expressed in milligrams of gallic acid equivalent per 100 ml (mgGAE/100 ml) of juice.

6.2.4.3 Determination of proteins

Total soluble protein was estimated following Lowry et al (1951) with minor modification. 5 ml of the juice sample was made up to 50 ml with phosphate buffer. Then 2 ml of 20% trichloroacetic acid was added to 2 ml of the mixture. After then, the mixture was left to settle for 30 minutes. The solution was then centrifuged for 25 minutes at 3000 rpm, rinsed with acetone, then centrifuged again. The solid was dissolved in 5 ml NaOH (0.1 N). 1 ml of the above solution was mixed with freshly prepared 5 ml alkaline copper sulphate reagent. After 1 minute, 0.5 ml Folin's reagent was mixed to it and kept undisturbed for 30 minutes. The absorbance was recorded at 660 nm with a blank containing 1 ml NaOH (0.1 N). The standard used for the test was Bovine serum albumin (BSA).

6.2.4.4 Determination of Ascorbic acid

The ascorbic acid of mandarin juice sample was determined following Sawhney and Singh (2005). The juice sample was diluted 10 folds with 6% metaphosphoric acid. From the diluted solution, 25ml was taken and titrated against 6-dichlorophenol indophenol solution until a faint permanent pink colour appeared.

6.2.4.5 Determination of Flavonoid content

The total flavonoid content was measured following Quettier et al (2000) with slight modification. The juice sample (1 ml) was mixed with 2% $AlCl_3$ (1 ml). The absorbance was measured at 430 nm after 10 minutes against a blank sample consisting of 1 ml sample solution with 1 ml of distilled water without $AlCl_3$. The total flavonoid content was evaluated using standard curve of rutin at 10-100 µg/ml. Each sample's total flavonoid content was represented in milligrams of rutin equivalent per 100 ml (mg RE/100 ml) of juice.

6.2.4.6 Ferric ion reducing assay

The reducing powers of mandarin juices for different pollination treatments were estimated following Oyaizu (1986) with minor modification. An aliquot of 1 ml of juice was mixed with 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide and incubated at 50°C for 20 minutes. The reaction was stopped by adding 2.5 ml of 10% trichloroacetic acid and left undisturbed for 10 minutes at room temperature. 2.5 ml of the upper portion of the solution was taken in a test tube and the same volume of distilled water was added along with 0.5 ml of $FeCl_3$. Thereafter, the absorbance was estimated at 700 nm. A greater absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid is used as a reference compound.

6.2.4.7 DPPH radical scavenging activity

The scavenging activity of mandarin juice was estimated according to the procedure modified by Shimada et al (1992) with slight modification. An aliquot of 0.5 ml of juice was mixed with 2.9 ml of 100 µM DPPH (dissolved in 80% ethanol). The mixture was vigorously shaken and set aside for 30 minutes at room temperature in a dark room. The control sample contained DPPH solution and same volume of 80% ethanol. Absorbance was read at 517 nm by using a UV-visible spectrophotometer. The scavenging effect was calculated using the following equation:

Scavenging effect (%) = [(Absorbance of blank - absorbance of sample)]/ (absorbance of blank) x 100

6.2.4.8 Statistical analysis

Based on the observational data obtained the frequency of visits of pollinators was analysed by Turkey test using SPSS version 18, IBM Crop. Pearson correlation test was performed between the abundance of pollinators and the environmental parameters so obtained using R ver. 3.5.2. Further, multiple linear regression was performed using SPSS version 18, IBM Crop to predict the effect of environmental variables on the abundance of pollinators. Quantitative traits of fruits for different treatments were analysed by multiple analysis of variance MANOVA with Gabriel Multiple Range tests. Spearman's rank correlation coefficient was used to understand the direction of relationship between the viability of pollens and time.

The data for phytochemical analysis were expressed as the mean and standard deviation. The flavonoid, phenol, ascorbic acid, protein composition, and scavenging activity were analysed for variation between different treatments OP, CP, SP, AP, AT. An analysis of variance was carried out for each nutrient separately using SPSS, IBM. Where treatment was significant ($p < 0.05$), Tukey's HSD (honestly significant difference) was used to compare between treatments.

6.3 Results

6.3.1 Foraging activity (Orchard A)

The most dominant pollinator recorded during the observation was the common honeybee *Apis cerana* (Fig. 37). The flowers bloomed in an asynchronous pattern and *A. cerana* repeatedly visited the same flower. The flowers had a lifespan of three days after which the petals fell as soon as a pollinator sat on it. The foraging activity began at 8:00 h in the morning and the frequency of visits reached its peak between 10:00 h to 13:00 h in the afternoon (Fig. 38) followed by a gradual decline in the visitation until the foraging activities stopped after 18:00 h. A similar pattern of foraging activity with a significant difference in the frequency of visits to the flowers ($r=0.04$, $p < 0.05$) was observed during the study period.

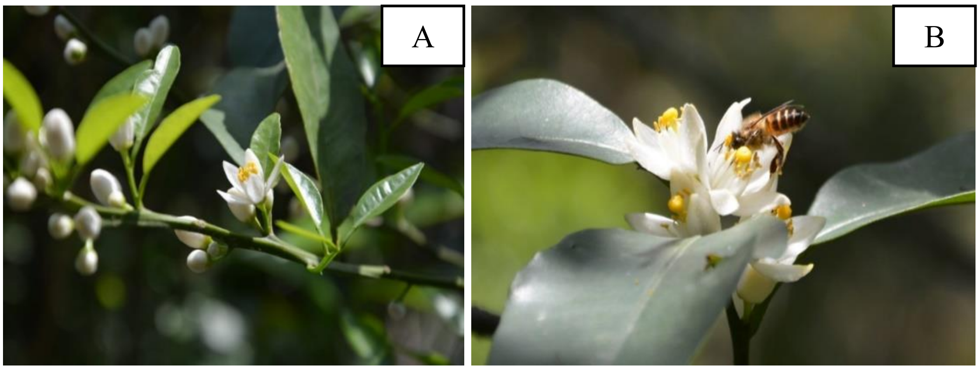


Fig. 37. Flower of mandarin orange **(A)**, and *A. cerana* pollinating mandarin flower **(B)**

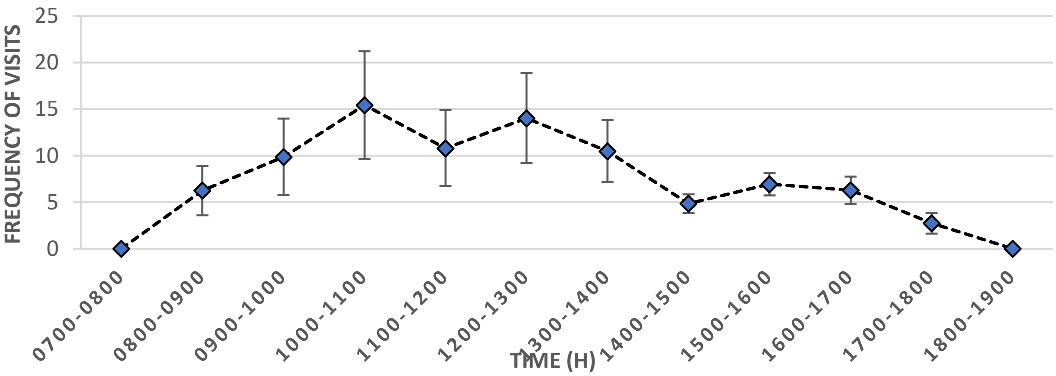


Fig. 38. Frequency of visits with respect to time slots by *A. cerana* at different times of the day over a study period of 7 days, total number of flowers in the study plot (n): 30

6.3.2 Linking pollinator abundance with environmental variables

Analysis of data on the abundance of pollinators on mandarin orange bloom in relation to the environmental variables revealed temperature to have a significant effect (t = 3.20, *p* = 0.019) on the field activities of *A. cerana* (Table 10). A significant correlation was observed between relative humidity (RH) and carbon-dioxide (CO_2) (r = 0.964, *p* = 0.01) and a significant negative correlation between CO_2 and temperature (r = -0.829, *p*=0.01; Table 9). A multiple linear regression model was obtained with an adjusted R^2 value of 0.613. The model estimated an increase of 47 ± 15 individual visitors of *A. cerana* per hour with an increase of °C rise in temperature. Among the three predictor variables CO_2 was excluded from the model (t = 0.630, *p* = 0.552).

Table 9: Correlation of visits of *A. cerana* in relation to Temperature, RH, and CO_2

Pearson correlation	**Visits**	**Temp**	**RH**	**CO_2**
Visits	1	.114	.202	.312
Temp	.114	1	-.829	-.928
RH	.202	-.928	1	.964
CO_2	.312	-.829	.964	1

Temperature (Temp.), Relative Humidity (RH), Carbon-dioxide (CO_2)

Table 10: Multiple linear regression analysis to assess the influence of environmental variables on the abundance of *A. cerana*

Variable	**B**	**SE**	**t value**	***p* value**	**R^2**
Temp (°C)	46.95	14.67	3.20	0.019	0.613
RH	30.47	15.74	1.93	0.101	
CO_2	-0.82	1.31	0.63	0.552	

β = regression coefficient; SE = Standard Error, R^2 = coefficient of determination; RH = Relative Humidity, Temp = Temperature, CO_2 = Carbon-dioxide

6.3.3 Fruit set, breeding system and pollinator dependency

The flowers emitted a strong scent which is a determinant factor in attracting the pollinators. Mandarins were found to be partially self-compatible (ISI = 0.28) with a pollinator dependency of 42%. The fruit set in open-pollinated flowers was higher compared to the supplemented ones. Fruit set in open pollination was 46% while the fruit set in autogamy and apomixis was 4% and 5% respectively (Fig. 39). The pollen limitation was estimated to be 0.30 with the pollination efficiency of *A. cerana* was empirically calculated to be 0.52. The potency of pollen declined with time with its fertility being 90.77, 55.56, 40.24, 28.85, 17.65 (%) at 0, 3, 6, 9, 24 ($r=0.001$, $p< 0.005$) hour respectively (Fig. 40).

6.3.4 Physico-chemical analysis

A significant difference was observed in the weight, height, diameter, and number of seeds between different treatments (Table 11) where the average weight of fruits was found to be the highest in open-pollinated (54.65 g) followed by geitonogamy (51.61 g) and apomixis (47.56 g). The fruit weight (22.56g), height (31.25g), and seed (5) were found to be the lowest in autogamy.

Overall, fruits resulting from open and supplementary pollination were heavier than those resulting from autogamy or apomixis (Table 11). For the remaining fruit variables, TSS and TSS/Acidity did not differ between pollination treatments (Table 11).

Table 11: Physico-chemical characters of fruits obtained from different pollination treatments

Treatments	Fruit variables						
	Av. Weight (g)	**Av. Height (mm)**	**Av. Diameter (mm)**	**No. of seeds**	**TSS (°Brix)**	**Acidity**	**TSS/Acidity**
Open pollinated	54.29 ± 2.23^{a}	41.44 ± 0.80^{a}	48.74 ± 1.51^{a}	12 ± 0.83^{a}	10.4 ± 1.81^{a}	1.13 ± 0.08^{b}	9.20 ± 1.67^{a}
Cross pollinated	40.78 ± 2.26^{d}	36.28 ± 1.02^{bc}	42.08 ± 1.14^{b}	10 ± 0.83^{a}	10.8 ± 0.84^{a}	1.10 ± 0.05^{b}	9.81 ± 1.40^{a}
Geitonogamy	51.56 ± 1.01^{b}	39.08 ± 1.05^{ab}	47.96 ± 1.25^{a}	10 ± 0.83^{a}	10.8 ± 1.30^{a}	1 ± 0.11^{c}	10.8 ± 1.88^{a}
Autogamy	22.68 ± 1.75^{e}	31.40 ± 1.75^{d}	35.49 ± 1.27^{c}	5 ± 1.30^{b}	11.1 ± 1.90^{a}	1.19 ± 0.07^{a}	9.32 ± 1.94^{a}
Apomixis	47.81 ± 1.99^{c}	38.20 ± 1.99^{b}	46.77 ± 1.86^{a}	7 ± 0.83^{c}	11.2 ± 1.48^{a}	1.18 ± 0.08^{ab}	9.49 ± 0.66^{a}
Tukey Post-Hoc test ($p<0.05$)	0.00	0.00	0.00	0.00	0.92 (*ns*)	0.01	0.45 (*ns*)

*P < 0.05, means with the same superscript within each row do not differ from each other

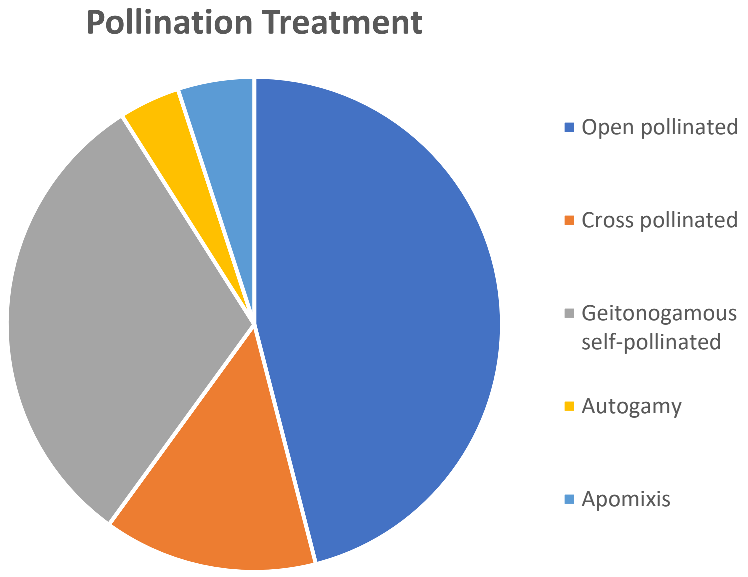


Fig. 39. Percentage proportion of *A. cerana* visitors on mandarin bloom

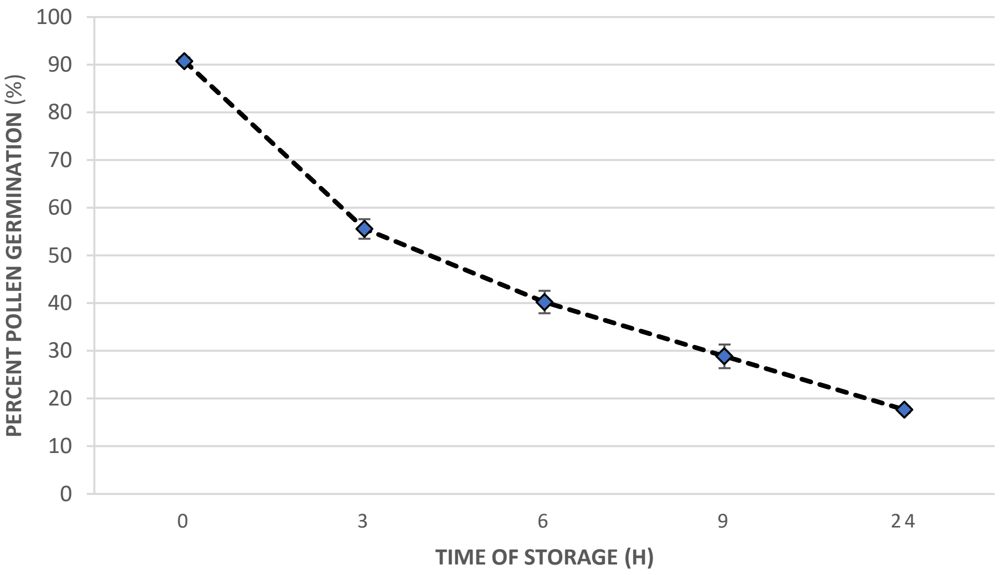


Fig. 40. Percentage of pollen fertility at different time storage

6.3.5 Foraging activity (Orchard B)

The flowers of Darjeeling mandarin bloomed for three days after which the petals dropped leaving only the gynoecium of the flower behind. The flowers were observed to bloom in an asynchronous fashion which was visited repeatedly by *A. cerana*. The most dominant pollinator observed throughout the observation was the common honeybee *A. cerana* (Fig. 41). The foraging began between 7:00 h – 8:00 h in the morning which reached its zenith at 11:00 h in the morning (Fig. 42) followed by a gradual decline till 18:00 h. The foraging activity by *A. cerana* followed a similar trend with no significant difference in frequency (r=0.99, $p<0.05$) throughout the observation period.

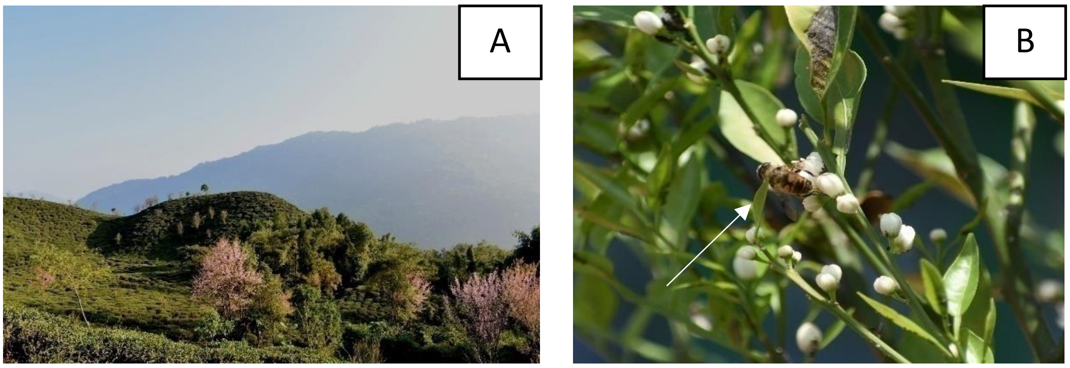


Fig. 41. An overview of Land cover around Orchard B **(A)**, and *A. cerana* pollinating mandarin flower **(B)**

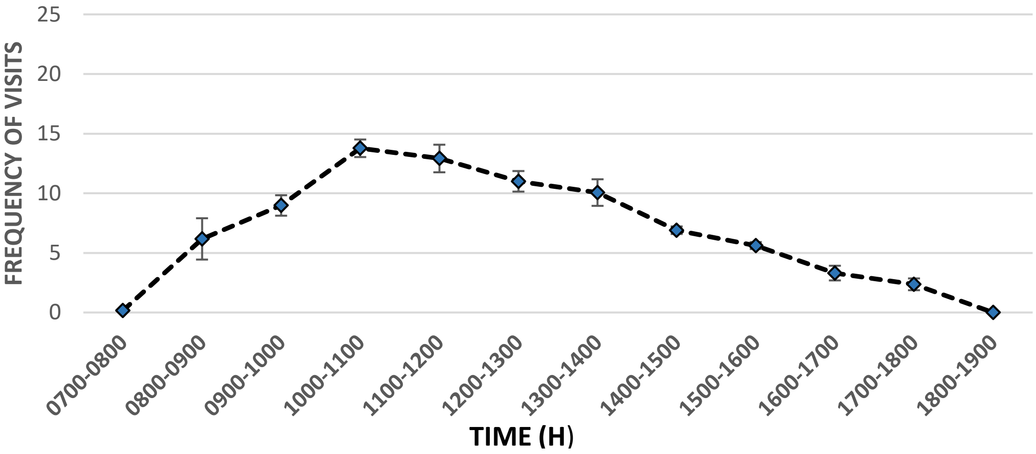


Fig. 42. Frequency of visits with respect to time slots by *A. cerana* at different times of the day over a study period of 7 days, total number of flowers in the study plot (n): 30

6.3.6 Linking pollinator abundance with environmental variables

Analysis of data on the abundance of pollinators on mandarin orange bloom in relation to the environmental variables revealed that relative humidity, temperature, and carbon dioxide had a significant effect (t = 4.45; p = 0.03) on the field activities of *A. cerana* (Table 13). A significant correlation was observed between relative humidity (RH) and carbon-dioxide (CO_2) (r = 0.984, p =0.01) and a significant negative correlation between CO_2 and temperature (r = -0.885, p=0.01) (Table 12). A multiple linear regression model was obtained with an adjusted R^2 value of 0.754 (Table 12). The model estimated an increase of 44 ± 10 individual visitors of *A. cerana* per hour with an increase of °C rise in temperature.

Table 12: Correlation of visits of *A. cerana* in relation to Temp, RH, and CO_2

Pearson correlation	**Visits**	**Temp**	**RH**	**CO_2**
Visits	1	.179	.149	.179
Temp	.179	1	-.841	-.885
RH	.149	-.841	1	.984
CO_2	.179	-.885	.984	1

Temperature (Temp.), Relative Humidity (RH), Carbon-dioxide (CO

Table 13: Multiple linear regression analysis to assess the influence of environmental variables on the abundance of *A. cerana*

Variable	**B**	**SE**	**t_{value}**	***p* value**	**R^2**
Temp (°C)	43.69	9.81	4.45	0.003	0.754
RH	-19.27	8.14	-3.37	0.05	
CO_2	2.56	0.74	4.48	0.01	

β = regression coefficient; SE = Standard Error, R^2 = coefficient of determination; RH = Relative Humidity, Temp = Temperature, CO_2 = Carbon-dioxide

6.3.7 Fruit set, breeding system and pollination dependency

The flowers emitted a strong scent which is a determinant factor in attracting the pollinators. Mandarins were found to be partially self-compatible (ISI = 0.30) with a pollinator dependency of 45%. The fruit set was higher in open-pollinated flowers than the supplemented ones. The fruit set in open pollination was 43% while the fruit set in autogamy and apomixis was 2% and 4% respectively geitonogamy (35%), cross-pollination (16 %) (Fig. 43). The pollen limitation was estimated to be 0.36 while the pollination efficiency of *A. cerana* was empirically calculated to be 0.55. The pollination efficiency of the pollen fertility declined with time with its fertility being

92.34, 58.36, 45.04, 29.23 and 19.22 (%) at 0, 3, 6, 9, 24 (r=0.001, $p < 0.05$) hour respectively (Fig. 44).

6.3.8 Physico-chemical analysis

A significant difference was observed in the weight, height, and the number of seeds between different treatments whereas no difference was observed in the acidity of the fruits. The average weight of fruits was found to be the highest in open-pollinated (59.33 g) followed by geitonogamy (52.12 g) and cross-pollinated (48.64 g). The fruit weight (31.71 g), height (27.40 mm), and seed (6) were found to be the lowest in autogamy. For the remaining fruit variables, acidity and TSS/Acidity showed no difference between treatments.

Overall, fruit weight and size were significantly larger in open-pollinated and supplemented flowers than in apomixis and autogamy (Table 14). For the remaining fruit variables, acidity and TSS/Acidity showed no difference between treatments (Table 14).

Table 14: Physico-chemical characters of fruits obtained from different pollination treatments

Treatments	**Fruit variables**						
	Av. Weight (g)	**Av. Height (mm)**	**Av. Diameter (mm)**	**No. of seeds**	**TSS (°Brix)**	**Acidity**	**TSS/Acidity**
Open pollinated	59.33± 2.42^{a}	48.48 ± 3.82^{a}	52.34 ± 2.31^{a}	13 ± 1.30^{a}	10.4 ± 0.24^{a}	1.22 ± 0.26^{a}	8.52 ± 1.90^{a}
Cross pollinated	48.64 ± 2.83^{b}	41.61 ± 1.64^{ab}	44.12 ± 2.15^{a}	11 ± 1.14^{ab}	10.7 ± 0.16^{ab}	1.19 ± 0.10^{a}	8.99 ± 0.83^{a}
Geitonogamy	52.12 ± 1.58^{ab}	41.39 ± 1.61^{b}	46.46 ± 1.96^{b}	10 ± 1.14^{b}	10.8 ± 0.19^{b}	1.14 ± 0.15^{a}	9.47 ± 1.46^{a}
Autogamy	31.71 ± 1.51^{c}	27.40 ± 1.43^{c}	32.27 ± 1.77^{b}	6 ± 0.84^{c}	11.1 ± 0.19^{b}	1.18 ± 0.29^{a}	9.40 ± 2.54^{a}
Apomixis	43.31± 1.71^{c}	37.28 ± 1.92^{c}	40.04 ± 1.69^{c}	8 ± 1.30^{c}	11.2 ± 0.08^{c}	1.16 ± 0.26^{a}	9.65 ± 3.60^{a}
Tukey Post-Hoc test (*p*<0.05)	0.00	0.00	0.00	0.00	0.00	0.98 (*ns*)	0.84 (*ns*)

*P < 0.05, means with the same superscript within each row do not differ from each other

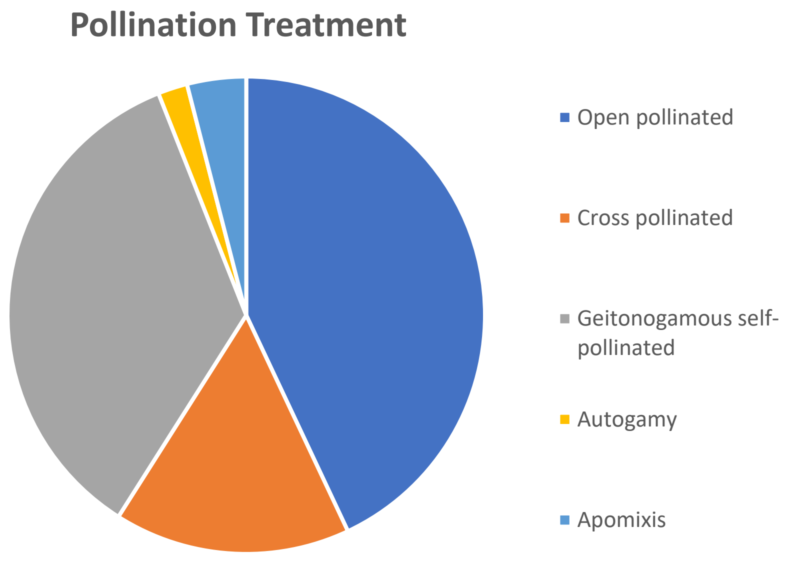
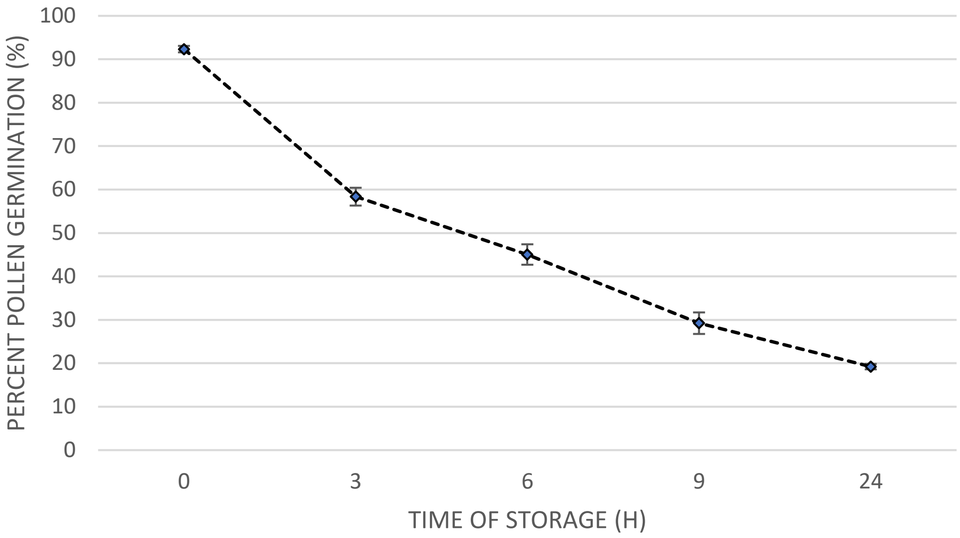


Fig. 43. Percentage proportion of *A. cerana* visitors on mandarin bloom



`**Fig. 44.** Percentage of pollen fertility at different time storage

6.3.9 Quantitative phytochemical estimation

The overall nutritional composition of the mandarin fruits differed between the treatment groups. This was confirmed by the multivariate analysis of variance (MANOVA) which found a significant difference in flavonoid content (r = 0.001, $p < 0.05$), phenolic content (r = 0.001, $p < 0.05$) and Vitamin C (r = 0.001, $p < 0.05$) (Fig 47c). However, the protein content of mandarin juices from different treatments did not differ (r = 0.520, $p < 0.05$) (Fig. 47d).

The DPPH scavenging activity and ferric ion reducing ability were highest in the fruits obtained from apomixis and lowest in fruits obtained from self-pollination (Fig. 45, 46). Similarly, the highest phenolic content and flavonoid content was in the apomixis fruits, the lowest flavonoid content in self-pollination, and the lowest phenolic content in fruits obtained from autogamy (Fig. 47 a, b).

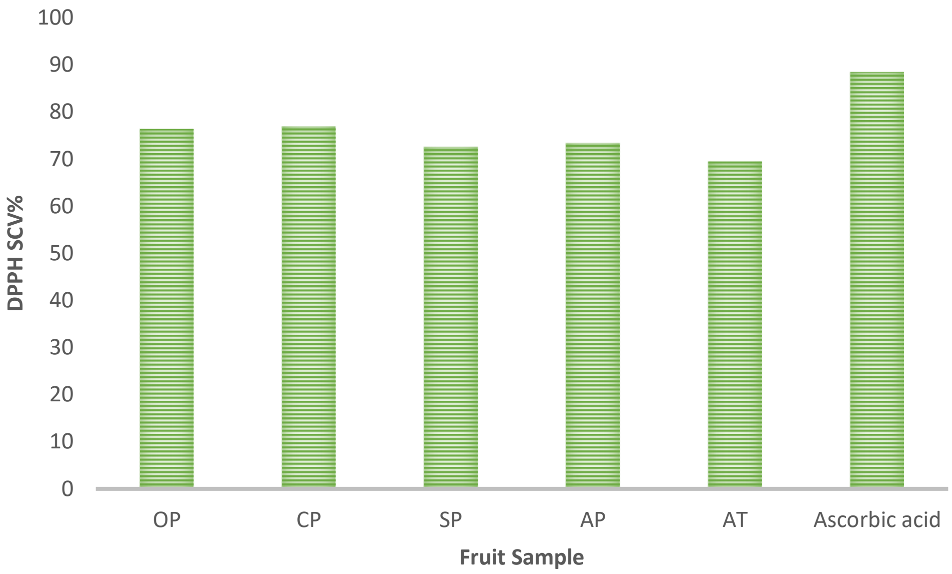


Fig. 45. DPPH free radical scavenging activity of fruit samples obtained from different treatments in comparison with standard Ascorbic acid. Values are expressed as the mean value ± standard deviation. OP – Open pollination, CP – Cross pollination, SP – Self- pollination, AP – Apomixis, AT – Autogamy

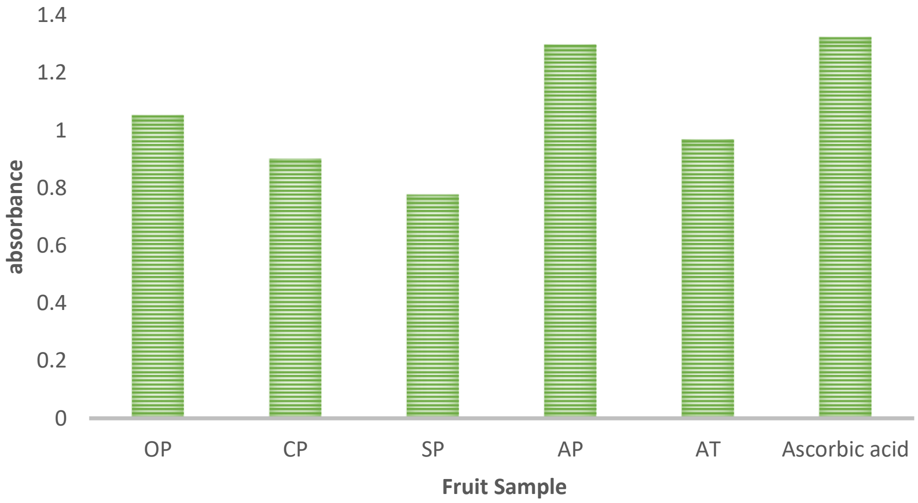


Fig. 46. Ferric ion reducing the ability of fruit samples obtained from different treatments in comparison with standard Ascorbic acid. Values are expressed as the mean value ± standard deviation. OP – open pollination, CP – Cross pollination, SP – Self-pollination, AP–Apomixis, AT–Autogamy

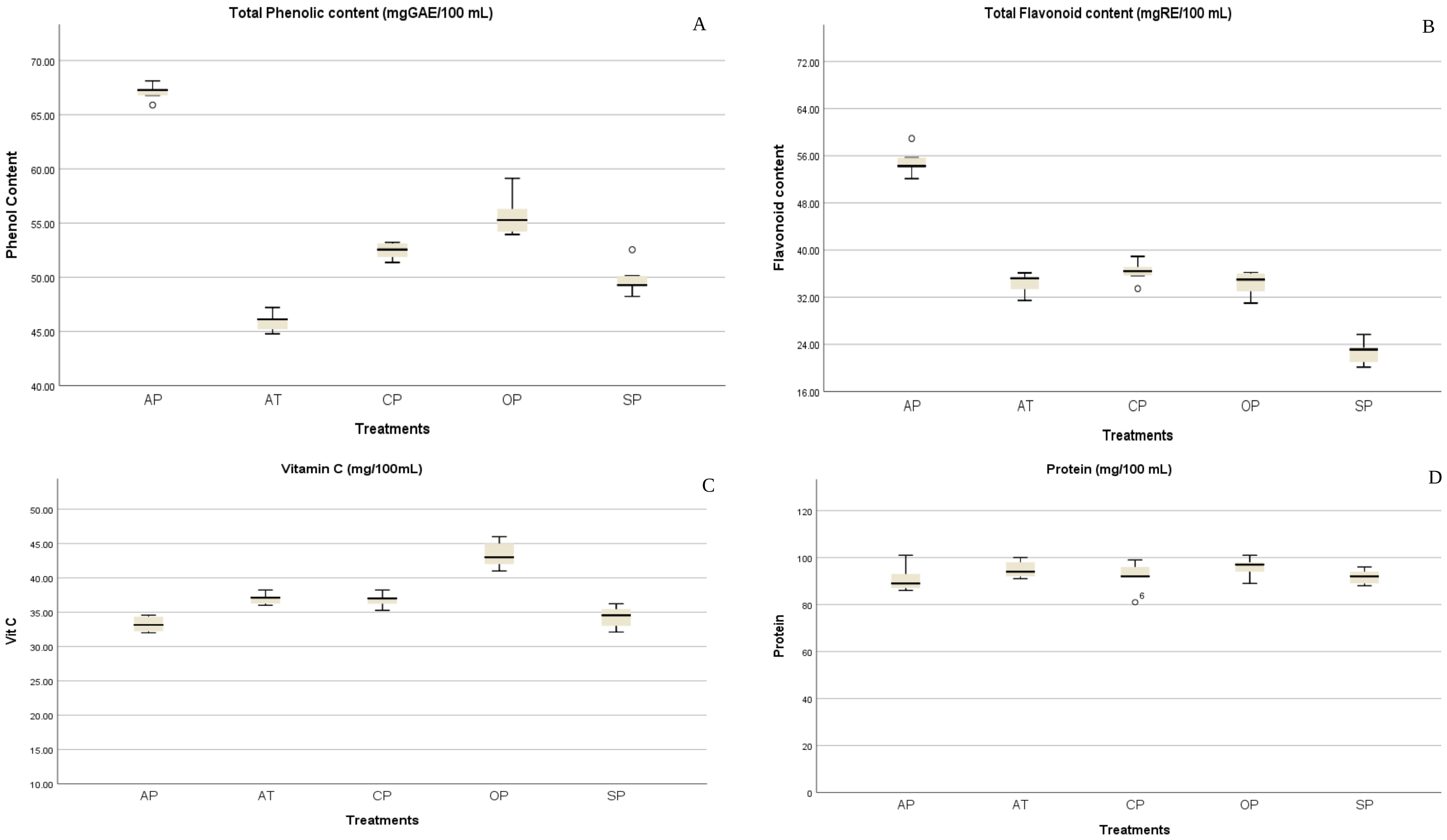


Fig. 47. The nutritional composition of mandarin juices from trees that received different pollination treatments. OP – open pollination, CP – Cross pollination, SP – Self-pollination, AP – Apomixis, AT – Autogamy

6.4 Discussion

Our results clearly indicate that the investigated mandarin in both Orchard A and Orchard B is partially self-compatible and is dependent on pollinators for its successful reproduction. This could be justified by the asynchronous flowering that mandarins have adopted. Flowering phenology has been considered an important factor in pollination success (Elzinga et al. 2007). As a result of the asynchronous flowering, a steady visit of pollinators is maintained throughout the flowering season. Asynchronous flowering has been reported to promote cross-pollination (Frankie and Haber 1983), reduce intraspecific competition for pollinators, and minimize effective population density (Bawa 1997; Primack 1980). To deal with the loss in the fertility of the pollens, the anthers also dehisce in an asynchronous fashion to ensure that an adequate number of viable pollens are available for cross-pollination. A similar decline in pollen fertility with time has been reported in Shogun (*C. reticulata*) in southern Thailand (Chelong and Sdoodee 2012). The regression coefficient reached statistical significance for temperature which appeared to be a significant predictor of the abundance of *A. cerana*. Gaira et al (2016) reported similar findings where the abundance of *A. cerana* rose during sunny days. Temperature has been reported to increase the visiting speed of honeybees with an increase in temperature (Benedek and Prenner 1972). We observed a positive influence of temperature in the abundance of *A. cerana* in both the study site. Similarly, a significant difference observed in the frequency of visits in Orchard A while a constant visit was observed in Orchard B can be attributed to the difference in temperature. Honeybees are reported to be more sensitive to poor weather conditions when compared to wild pollinators (Pomeroy and Fisher 2002; Vicens and Bosch 2000). Further, the difference in the land cover of the area where orchard A and orchard B is situated could be a driving factor in the observed difference in the abundance of pollinators. The diversity and patterns of land-cover and local management practices are known to be the main drivers structuring insect communities in crop systems (Kremen et al. 2007), wherein seminatural habitat acts as a source of flower-visitors for crops (Beduschi et al. 2015). We also demonstrated that open-pollinated, geitonogamy, and cross-pollinated flowers produced more fruits than the flowers bagged throughout anthesis and left untreated along with flowers that were emasculated and bagged. These findings strongly indicate that the investigated mandarin is dependent on pollens from other flowers thereby, establishing the importance of pollinators. Sanford (1992) reported that mandarin and its hybrids are self-incompatible and require pollination for its reproduction. Moreover, data from the fruits so obtained showed a significant difference in weight, diameter, height, and seeds of autogamous and open-pollinated fruits. Our findings are consistent with the breeding system experiments performed on Kinnow mandarin (Manzoorul-Haq et al. 1978). The effect of pollination on acidity and TSS is arguable. Although there was a significant difference in the physical attributes of the fruits obtained from various pollination treatments, the chemical attribute comprising of TSS and acidity of Sikkim mandarin and Darjeeling mandarin remained unaffected. Likewise, honeybee pollination improved the quantity and quality of sweet orange (*Citrus sinensis*) producing heavier, less acidic fruits (Malerbo-Souza et al. 2004). On the contrary to our finding, Wallace and Lee (1999) observed an improvement in the sugar content of pollinated fruits.

Although strawberries were reported to have a longer shelf life due to bee pollination (Klatt et al. 2014), the mechanisms for the impact of cross vs. self-pollination on nutrients is unexplored (Brittain et al. 2014). We found a significant difference in the phytochemical constituents from different treatments except for protein. Unlike previous studies that reported cross-pollinated fruits to possess the most favourable nutritional content (Brittain et al. 2014), we found a higher flavonoid, phenolic and antioxidant activity in fruits obtained by apomixis which places pollination as least needed for nutritional content in mandarins. However notable exception similar to our findings has been reported by Brittain et al (2014) where they reported a higher composition of vitamin E (α Tocopherol) in self-pollinated as compared to cross-pollinated almonds. Our study also suggests pollination to bear the least effect on the protein content of the fruit. Although it should be noted that the majority of *Citrus* amino acids are non-essential (Block and Bolling 1944) and likely make a small impact on human nutrition (Brittain et al. 2014).

Our observations showed that the most dominant and true pollinators of mandarin are social bees of the family Apidae: *A. cerana*. Out of the 24 species of insects reported, *A. cerana* was found to be the most dominant pollinator (Pradhan and Devy 2018). *A. cerana* landed on top of the flower and performed circular movements that allowed contact of its abdomen on the stigma. *A. cerana* also rubbed its thorax and abdomen on the stigma while it inserted its proboscis in the nectaries. *A. cerana* was observed to visit the same flower several times ensuring that adequate pollen is deposited on the stigma. *A. cerana* along with *A. dorsata, A. florea,* and *A. mellifera* were reported to pollinate mandarin oranges in Nepal (ICIMOD 2003). Pollinator-dependent plants suffer from a phenomenon called pollen limitation (Knight et al. 2005) due to the inadequacy of pollen reaching the stigmas. As a result, there is a decrease in the sexual reproductive output of plants (Vaissière et al. 2011). The weight and size of any horticultural crop are determinant characteristics to determine its economic value (Castro et al. 2021). In our study, open pollination produced heavier fruits in both the orchards which indicates the adequate presence of pollinators for a successful transfer of pollen. In particular, an abundance of pollinators can also be attributed to lower pollination deficits, while the abundance of *A. cerana* in both the orchards contributed to higher fruit set in open-pollinated flowers. Furthermore, the apparent efficiency of *A. cerana* could be explained by the effectiveness of specific pollinator species, as indicated by MacInnis and Forrest (2019) for strawberries, who discovered that bee visitation had the greatest effect on yield.

CHAPTER 7

Summary and Conclusion

7.1 Morphological assessment

A positive correlation was found between fruit weight and height (0.772), fruit diameter and weight (0.549), total soluble solids (TSS) /acidity and fruit diameter (0.473). The first 6 components of Principal component analysis (PCA) exhibited 69.34% of the total variation. DIVA-GIS showed the highest diversity index for fruit weight, fruit diameter and TSS/acidity in East district, Sikkim. The highest coefficient variation for fruit diameter was observed in the East district and Darjeeling district, WB and TSS/acidity and fruit weight in east district of Sikkim. The dendrogram generated divided the accessions into two major clusters – Sikkim and Darjeeling mandarin with a similarity coefficient of 0.49. The grid maps generated identified diverse accessions in the East district and Darjeeling district, which can be a source of superior germplasm.

7.2 Current understanding on identification and diversity

The present study demonstrated the existence of wide variation in morphological characters among the mandarin accessions collected from the four districts of Sikkim and two hilly districts of West Bengal. A wide variation and diversity were observed within and between the districts which suggests that there is a larger play of genetic variation along with environmental factors that trigger morphological variation. The genetic variation can also be confirmed by the variation in TSS among the fruits. Further, the morphological characters divided the accession into Sikkim mandarin and Darjeeling mandarin. *C. reticulata* is an important cash crop for the farmers in the Sikkim and Darjeeling Himalayas, India. Inadequate information has resulted in a lack of understanding of the region's varieties. In the present time where mandarin cultivation and its production in Darjeeling and Sikkim Himalayas has become an issue of concern, our study helps in correct identification and classification for future specific breeding purposes, detect mislabelled accessions, understand the genetic variability that the collection represents in order to increase or maintain an appropriate range of genetic diversity. The fruit attributes such as weight, height, and diameter characterized Sikkim mandarin while TSS/Acidity and the flower attributes characterized Darjeeling mandarin. The horticultural traits such as fruit weight and diameter that are considered important for marketability were prominent in east district of Sikkim and Darjeeling district of West Bengal. Further, fruit and flower characteristics were identified be a determinant factor in selecting superior varieties that can act as parents for breeding purposes. Further, the germplasm identified in our study with the lowest number of seeds could fulfil the aim of *Citrus* breeders to develop seedless, easy-peeling fruits. Therefore, exploitation of potential germplasm from such areas will not only help in crop improvement but also aid in reviving the *Citrus* industry.

7.3 Floral Biology

Himalayan *Citrus* exhibits wide variation in the Northeastern Himalayan region of India. Among the *Citrus,* mandarin is considered to be highly heterogeneous due to somatic mutation, propagation through seed or cross-pollination. Furthermore, domestication over the years has resulted in the formation of hybrids. As a result, the genus' taxonomy and systematics have been reported to be complicated. Besides the morphological assessment, study of palynological characters have been identified as an important tool for solving taxonomic problems. As discussed in Section 7.1 the cultivars of Sikkim and Darjeeling are least understood and therefore a novel approach must be adapted to draw relationship among mandarins grown in Sikkim and Darjeeling Himalayas using pollen morphological characters. According to our study, pollens play an important role in bringing about variations in mandarins. The quantitative characters of pollen showed significant variation among the pollens of the accessions collected from several locations. PCA divided the accessions from Darjeeling in a separate quadrant largely governed by equatorial diameter of pollens while pollens of Sikkim and Kalimpong were concentrated in the other quadrant whose variation was determined by pollen length, colpi length and ratio of pollen length to equatorial diameter. Therefore, palynological investigations indicated that variations in pollen morphological characters are of taxonomic importance. The pollen size of all the accessions belonged to the group media (diameter 25-50 µm) which indicates that the flowers of *C. reticulata* are pollinated by honeybees where the wind is not a major factor. While we observed the pollen shapes of our accessions to be prolate or sub-prolate there are studies reporting pollen shapes of *C. reticulata* to be circular-elliptical or prolate-spheroidal. This confirms another assertion that mandarin is the most diverse group of *Citrus* species and consists of numerous intergeneric and interspecific hybrids.

The studied accessions exhibited similar aperture and exine ornamentation which confirms the assertion that *C. reticulata* is an ancestral species from where other hybrids were derived. Similar to the assessment of morphological characters that determined mandarin accessions to be genetically different resulting in morphological diversity pollen characters contributed similarly in confirming the assertion that the mandarin of Sikkim and Darjeeling are diverse.

7.4 Pollination Biology

The adaptation of a fruit crop to exhibit selfing, outcrossing, apomixis or mixed mating strategies is considered a mechanism to ensure successful reproduction irrespective of unpredictable pollinator availability. The dwindling production of mandarins has been attributed to pest and disease infestation, lack of research understanding of breeding system and pollination biology of mandarins grown in eastern Himalaya, India. A pertinent question that needs to be answered is whether pollinator plays any role in the yield of mandarins grown in eastern Himalayas and how it has adapted to the plant-pollinator interaction. In order to ascertain the breeding system of the plant the flowers were divided into five sets to test for xenogamous pollination, geitonogamous self-pollination, open pollination, autogamy and apomixis respectively. *Apis cerana* was observed to be the most dominant pollinator of mandarins. Field experiments showed mandarins to be partially self-incompatible (index of self-incompatibility (ISI) = 0.28) in Orchard A and 0.32 in Orchard B. The fruit set in Orchard A in open pollination was 46%, geitonogamous self-pollination (31%), cross pollination (14%), autogamy (4%) and apomixis (5%). The fruit set in Orchard B in open pollination was 43%, geitonogamous self-pollination (35%), cross pollination (16%), autogamy (2%) and apomixis (4%). The pollination efficiency of *A. cerana* was estimated to be 0.52 and 0.55 in Orchard A and Orchard B respectively which establishes it as an effective pollinator. The average weight of fruits was highest in open pollination (54.65 g), geitonogamous self-pollination (51.61 g) and lowest in autogamy (22.56 g) in Orchard A. Similarly, the average weight of fruits was highest in open pollination (59.33 g), geitonogamous self-pollination (52.12 g) and lowest in autogamy (31.71 g). The results suggest that mandarins are dependent on pollinators for successful fruit set and better fruit quality.

7.5 Pollination of mandarin: Is it important?

In summary, we investigated the breeding system, pollination biology of mandarin (*C. reticulata* Blanco) grown in the eastern Himalaya, India. The breeding system is characterized as partially self-compatible as a result, the flowers opened asynchronously to ensure geitonogamous or cross pollen transfer. This ensured geitonogamy, cross-pollination and open pollination to yield greater fruit set with improved fruit quality. Further, *A. cerana* was recorded to be the dominant pollinator. The lack of pollen limitation unveiled the efficiency of *A. cerana* in pollinating mandarin flowers whose abundance was positively influenced by the rise in temperature. The physical attribute of the fruits obtained from various treatments were significantly affected by pollination than the chemical attributes. There was also a significant difference in the phytochemical constituents from different treatments except for the composition of proteins. However, the antioxidant activity was observed to be higher in fruits obtained from apomixis. Finally, we confirm that mandarins are dependent on pollinators and is important not only for higher yield but also for better fruit quality. We also confirm that *A. cerana* is an efficient pollinator for mandarin and their abundance as a pollinator were adequate enough for greater fruit set. However, the importance of pollination for the additional nutritional benefit remains doubtful.

Although the major pollinators for mandarins grown in Sikkim and Darjeeling Himalayas are known, the quantitative relationship of pollinator abundance, activities and densities with plant and the resultant fruit set are largely unknown. The present study thus highlighted the importance of pollinators in the production of mandarin which has been neglected for a very long time. The findings of this study could be used for conservation, imaginative approaches to management and above all creating awareness among the mandarin farmers about the importance of pollination and the functionality of pollinators.

In **Chapter 5** we inferred with the aid of palynological studies that the mandarins cultivated in Darjeeling and Sikkim Himalayas are ancestral species. Further, in Chapter 4 we identified that the ancestral species have undergone variation. As a result, mandarins are of two types – Darjeeling mandarin and Sikkim mandarin. Mandarins are an important cash crop of Darjeeling and Sikkim Himalayas. However, the decline in its productivity has opened pandora box of questions for researchers that needs to be addressed. We have tried to address the issue by ascertaining the nuances of variation and classification of mandarins along with its breeding adaptation with respect to pollination. However, the list of future prospects with regards to our study are as follows:

1. **Molecular-marker based techniques analysis**

   Morphological or palynological assessment should always be complemented by assessment through molecular-marker based techniques to ascertain the difference in genetic variability among the mandarina accessions collected from Darjeeling and Sikkim Himalayas. This in turn would provide an opportunity to enhance the credibility of the study considering the fact that phenotypic characters are partial reflection of heritable genetic variability which is influenced by environment for its growth and development.

2. **Nutritional analysis**

   How pollination impacts nutritional composition has been addressed briefly in our study. However, an in-depth study with larger sampling will help in understanding this pressing question on relationship between nutrition composition and pollination.

3. Pollination studies

Mandarin is a highly heterogeneous group and pollination in mandarin is observed to be highly dynamic. Therefore, we recommend similar kind of study should be conducted in several parts of Darjeeling and Sikkim Himalayas to get a comprehensive understanding of the pollination requirements of mandarin.

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Publications in peer reviewed Journals

1. Aditya Pradhan, Arun Chettri, **Subhankar Gurung** (2015). Diversity and Distribution of Genus Dioscorea in Sikkim Himalaya: an application of DIVA-GIS. NeBIO 6 (3) 29-31
2. **Subhankar Gurung**, Arun Chettri, Aditya Pradhan (2019). Assessing semi-natural forests as population sink for pollinators in Mountainous agricultural field. Ecology, Environment and Conservation 25 (2) 713-717
3. **Subhankar Gurung**, Aditya Pradhan, Arun Chettri (2019). Pollination in an endemic and threatened monoecious herb *Begonia satrapis* C.B. Clarke (Begoniaceae) in the eastern Himalaya, India. Journal of Threatened Taxa 11 (10) 14328-14333 https://doi.org/10.11609/jott.4256.11.10.14328-14333
4. **Subhankar Gurung,** Arun Chettri (2019). Vegetation analysis of Oak Forests of Fambonglho Wildlife Sanctuary in Sikkim Himalayas. International Journal of Basic and Applied Biology 6 (3) 192-197
5. ***Subhankar Gurung**, Arun Chettri, Meera Tamang, Mamta Chettri (2020). Identifying morphological diversity and superior germplasm of *Citrus retiulata* Blanco (Mandarin) in Sikkim and Darjeeling Himalayas, India: an application of DIVA-GIS and cluster analysis. Australian Journal of Crop Science 14 (10) 1575-1582 https://doi.org/10.21475/ajcs.20.14.10.p2351
6. **Subhankar Gurung**, Arun Chettri (2021). Threat to *Citrus* in a Global Pollinator Decline Scenario: Current understanding of its Pollination Requirements and Future Directions [Online First]. IntechOpen https://doi,org/10.5772/intechopen.101159 Available from: https://www.intechopen.com/online-first/79341

(Publication in * symbol is included in thesis)

Seminar/Symposium/Conference

1. Participate in the **Asia-Pacific Youth Forum Towards Sustainable and Climate Resilient Mountain Livelihoods** held from 5 to 8 October, 2015 in Kathmandu, Nepal organized by the International Centre for Integrated Mountain Development through its Asia Pacific Mountain Network in partnership with the Himalayan University Consortium.
2. Presented a paper on a topic entitled **"Pollination: a Sexual Mimicry in a rare and endemic herb, *B. Satrapis* C.B. Clarke"** in the oral session in National Seminar on Understanding Himalayan Phylodiversity in a changing Climate, organized by Botanical Survey of India, Sikkim Himalayan Regional Centre, Gangtok from 9-10th March, 2017.
3. Delivered a talk on the topic entitled **"Identifying superior germplasm and morphological diversity of *Citrus reticulata* Blanco for breeding purposes in Sikkim on Darjeeling Himalayas"**in the oral session in International Conference on Recent Trend and Practices in Science, Technology, Management and Humanities for Sustainable Rural Development (STMH-2019) organized by Department of Rural Development, University of Science and Technology, Meghalaya from 6th – 7th September, 2019.
4. Participated in a training workshop on **"Measuring Ecosystem Services in Darjeeling-Sikkim Himalaya"** organized by Ashoka Trust for Research in Ecology and the Environment (ATREE) at the Divya Vani Centre, Loreto Convent Road, Darjeeling (W.B) from February 19th to February 21st 2015.
5. Delivered a talk on the topic entitled **"How efficient is *Apis cerana* in pollinating mandarin orange (*Citrus reticulata* Blanco)?: Pollination behaviour, pollination efficiency and impact on pollination"** in the oral session in International Conference on "Chemical Ecology, Environment and Human Health: Emerging frontiers and Synthesis (ICCEEHH 2019)" organized by Department of Zoology, Sikkim University on August 9-10, 2019.
6. Participated in a training workshop on **"Data Management and Analysis for Biologists using R"** held during 25th February to 9th March 2019, organized by Department of Zoology, Sikkim University, Gangtok in collaboration with Ashoka Trust for Research in Ecology and he Environment (ATREE).

7. Participated in two days online workshop on **“Species distribution modelling using MAXENT and ENMtools”** organized by Biologia Life Science LLP from 12-13 February, 2021.
8. Participated in four days online workshop on **“Multivariate data analysis using PAST”** organized by Biologia Life Science LLP from 16-19 February, 2021.
9. Participated and completed an online course on **“Basics of Ecological Niche Modeling”** held between 19th and 20th June, 2021 organized by ChaturUllu Lab.

Australian Journal of
Crop Science
AJCS
AJCS 14(10):1575-1582 (2020) ISSN:1835-2707
doi: 10.21475/ajcs.20.14.10.p2351

Identifying morphological diversity and superior germplasm of *Citrus reticulata* Blanco (Mandarin) in Sikkim and Darjeeling Himalayas, India: an application of DIVA-GIS and cluster analysis

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Abstract

Citrus reticulata is an important cash crop for the farmers in the Sikkim and Darjeeling Himalayas, India. The lack of knowledge of its diversity has only resulted in the lack of uniformity in the fruit quality. It has become imperative to identify superior varieties that meet the demands of the market to improve the citrus industry. Hence, a total of 105 accessions of mandarin were collected randomly from different locations to assess the morphological diversity using cluster analysis and DIVA-GIS. The orchards were randomly visited in each district of the state of Sikkim and two hilly districts of the state of West Bengal (WB). A sample tree was selected from each orchard and the quantitative and qualitative characters of its leaves, flowers, and fruits were measured. A significant variation was observed in the quantitative characters with a positive correlation between fruit weight and length, fruit diameter and weight, total soluble solids (TSS) /acidity and fruit diameter. The first 6 components of Principal component analysis (PCA) exhibited 69.34% of the total variation. DIVA-GIS showed the highest diversity index for fruit weight, fruit diameter and TSS/acidity in East district, Sikkim. The highest coefficient variation for fruit diameter was observed in the East district and Darjeeling district, WB and TSS/acidity and fruit weight in East district. The dendrogram generated divided the accessions into two major clusters. The grid maps generated identified diverse accessions in the East district and Darjeeling district, which can be a source of superior germplasm.

Keywords: *Citrus reticulata*; morphological; germplasm; quantitative; diversity; DIVA-GIS.
Abbreviations: DIVA-GIS_Data-Interpolating Variational Analysis – Geographical Information System.

Introduction

Citrus belongs to the family, Rutaceae and is one of the most important cash crops in the world (Swingle and Reece, 1967). The origin of *Citrus* cannot be determined but reports suggest that it originated from the south and southeast tropical regions of Asia (Moore, 2001; Sharma et al., 2004; Ladaniya, 2008; Singh, 2010). The natural and commercially cultivated citrus include sweet orange, mandarin, lemons and lime. The North-Eastern part of India is a treasure house of citrus species and their varieties (Sharma et al., 2004) where 11.7% of its total area is under its cultivation (NHB, 2006). Among all the citrus fruits, mandarin *Citrus reticulata* Blanco, is an important cash crop and constitutes about 41% of the total citrus fruits that are produced in India (Das et al., 2005). The North-Eastern part of India comprises of diverse forms of mandarin and could be considered as its center of origin (Ray and Deka, 2000). Mandarin is the most important cash crop in Sikkim (Kishore et al., 2010) and the adjoining Darjeeling hills. Sikkim alone contributes 2% of the total citrus production and covers an area of 5% (NHB, 2006). Mandarin is mainly grown in the lower hills of Sikkim and Darjeeling Himalayas under sub-tropical humid climatic conditions (Kishore et al., 2010). Anecdote from the farmers of mandarin suggests that mandarin is cultivated for several decades. Unfortunately, due to the lack of data and knowledge about the varieties that their forefathers sowed in the field, very little is known about mandarin varieties and their relatives. This has resulted in Sikkim mandarin being referred to as Darjeeling mandarin and vice versa (Gurung et al., 2017). There are several reports which suggest that *C. reticulata* Blanco comprises of ecotypes which include Darjeeling mandarin and Khasi mandarin (Das et al., 2004), while there are certain reports that suggest *C. reticulata* comprise of Sikkim mandarin as well (Kishore et al., 2010).

Therefore, in order to clear this confusion, it is imperative to closely observe the morphological characters of the cultivated mandarin species and derive a relationship between them. Furthermore, natural hybridization and spontaneous mutations are a common phenomenon in *Citrus* spp. (Dorji and Yapwattanaphun, 2011) which has led to the diversification of the species over the years. The evidence of the occurrence of zygotic twins (Das et al., 2007) attributes to greater variation and lack of uniformity in fruit quality in the plant (Dorji and Yapwattanaphun, 2011). The level of diversity that exists is

Chapter

Threat to *Citrus* in a Global Pollinator Decline Scenario: Current Understanding of Its Pollination Requirements and Future Directions

Subhankar Gurung and Arun Chettri

Abstract

Pollinators are vital for world biodiversity and their contribution to agricultural productivity is immense. Pollinators are globally declining with reports such as colony collapse being documented. *Citrus* exhibits a varying degree of pollination requirements due to its vast cultivars being developed all the time. The article intends to understand the breeding system of a few commercially important *Citrus* groups and discern its dependency on pollination services. The threat related to pollinator decline to the *Citrus* industry is measured not only by its reliance on pollinators but also the requirement of the consumers and manufacturers who mostly seek seedless varieties. Therefore, the threat can be tackled by developing high-quality seedless varieties where pollination requirement is absent. Although the importance of pollinators on several self-incompatible varieties cannot be negated, the impact of pollinator decline on its production will entirely depend upon the demand of the market.

Keywords: breeding system, pollinators, pollination, *Citrus*, fruit set

1. Introduction

Pollinators are insects or vectors that transfer the pollen from the male part of the flower, i.e., anther to the female part of the flower i.e., stigma enabling fertilization and seed production [1, 2]. Pollination is an ecosystem service that enhances crop production and helps in sustaining human life [1, 3]. Furthermore, many wild plants that provide calories and micronutrients to human diets require pollination as well [4]. Approximately, 90% of wild flowering plant species worldwide rely on insect vectors for pollen transmission. These plants are essential for ecosystems to function properly because they supply food, habitat, and other resources to a variety of different species [5]. One-third of the agricultural plants, including the cultivars, are dependent on animal pollinators for their reproduction and increased fruit set [6]. Bees play a pivotal role among pollinators by visiting more than 9% of the leading global crop plants [3]. Unfortunately, agricultural intensification has led to a loss of habitat of many insect pollinators and monoculture plantings have

Journal of Threatened Taxa | www.threatenedtaxa.org | 26 August 2019 | 11(10): 14328–14333

COMMUNICATION

ISSN 0974-7907 (Online)
ISSN 0974-7893 (Print)

POLLINATION IN AN ENDEMIC AND THREATENED MONOECIOUS HERB *BEGONIA SATRAPIS* C.B. CLARKE (BEGONIACEAE) IN THE EASTERN HIMALAYA, INDIA

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Abstract: *Begonia satrapis* was studied for its pollination aspects at Sumbuk, Sikkim, India. The floral details and the foraging behaviour of insects visiting the flowers were examined to define the pollination syndrome and its functionality for the success of sexual reproduction in this species. The flowers do not produce nectar and offer only pollen as floral reward to foraging insects. Therefore, male flowers were foraged more for its pollen than the female flowers. There was a significant difference in the visit to male and female flowers by both *Apis florea* and *Bombus breviceps*, respectively. The bees spent more time on male flowers than on female flowers. The bees appear to rely on visual stimuli to visit male and female flowers. The plant produces abundant fruit and seed set in both hand and open-pollinations indicating that it is facultatively xenogamous. The female flowers lacking any reward resemble male flowers and in effect are pollinated by deceit.

Keywords: *Apis florea*, *Bombus breviceps*, deceit, northeastern India, Sumbuk.

***Nepali* सार:** भारतको उत्तर-पूर्वीय राज्य सिक्किमको सुम्बुकमा पाइने मंगरकाँचे (*Begonia satrapis*)को परागण प्रक्रिया मार्गको विशेष अध्ययन हो। यहाँ पाइने यस प्रजातिको प्रजनन प्रक्रिया तथा स्त्री फूल अनि नर फूलको पराग मिलन प्रक्रियालाई बुझ्न, फूलको सुक्ष्म भागको जाँच साथै फूलको पराग खान आउने किटपतङ्गको क्रिया कलापमाथी विशेष जाँच गरिएको छ।फूलमा रस नहुनाले गर्दा किटपतङ्गले परागको धुलोलाई नै टिप्ने गर्दछ। विशेष गरि नर फूलमा परागको धुलो पाइनाले गर्दा माँरी (*Apis florea*) अनि भमरा (*Bombus breviceps*)ले नर फूलमा मात्र अधिक समय बिताएको पायौं।आफ्नै हातले गरिएको परागण प्रक्रिया साथै प्राकृतिक रुपबाटले हुने परागण (Open pollination)द्वारा अधिक मात्रामा फल र बिउ लागेको हुनाले मंगरकाँचेको यस प्रजातिलाई *फ्याकल्टेटिभ जिनोग्यामस्* भन्न सकिन्छ। माँरी अनि भमराले आफ्नो नजरमाथी भर परेर नै नर फूल र स्त्री फूलमाथी बसेको अवलोकन गर्यौं। स्त्री फूलमा किटपतङ्गको निम्ति कुनै रस अथवा आहार नभए पनि नर फूल झैं दुरुस्त देखिने हुनाले किटपतङ्ग झुक्किएर स्त्री फूलमा बस्दा नर फूलबाट टिपेको परागको धुलो छरिन्छ, यसो हुँदा फूलमा परागण प्रक्रिया सम्भव भएको पाइन्छ।

DOI: https://doi.org/10.11609/jott.4256.11.10.14328-14333

Editor: A.J. Solomon Raju, Andhra University, Visakhapatnam, India. **Date of publication:** 26 August 2019 (online & print)

Manuscript details: #4256 | Received 15 May 2018 | Final received 31 July 2019 | Finally accepted 05 August 2019

Citation: Gurung, S., A. Pradhan & A. Chettri (2019). Pollination in an endemic and threatened monoecious herb *Begonia satrapis* C.B. Clarke (Begoniaceae) in the eastern Himalaya, India. *Journal of Threatened Taxa* 11(10): 14328–14333. https://doi.org/10.11609/jott.4256.11.10.14328-14333

Funding: This research has been funded by the Department of Biotechnology (DBT), Government of India (Project No. BT/Env/BC/01/2010).

Competing interests: The authors declare no competing interests.

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Author contribution: Study designed by SG; Data collected by SG, AP; analysis of data done by AC, and SG wrote the manuscript.

Acknowledgements: The authors would like to thank the Department of Botany, Sikkim University for providing all the logistical support and laboratory facilities for this research. Our sincere thanks go to Dr. M.P. Thapa, guest faculty and Sailendra Dewan, research scholar, Department of Zoology and Dr. Somnath Dey, Department of Zoology, Darjeeling Government College for their assistance in helping us identify the pollinators. Lastly, we would like to thank the villagers for their cooperation and hospitality at Sumbuk.

International Journal of Basic and Applied Biology
p-ISSN: 2394-5820, e-ISSN: 2349-5839, Volume 6, Issue 3; July-September, 2019, pp. 192-197
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http://www.krishisanskriti.org/Publication.html

Vegetation analysis of Oak Forests of Fambong lho Wildlife Sanctuary in Sikkim Himalayas

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Abstract—*A total of 4683 plants belonging to 62 families, 92 genera were enumerated from the study site. The topmost canopy was formed by Quercus lineata, Lithocarpus pachyphyllus, Quercus lamellosa, Castanopsis tribuloides while the second layer was formed by Symplocos lucida, Caruga pinnata. The highest adult tree species were recorded of Elaeocarpus sikkimensis (119 ind/ha) followed by Daphne sp. (56 ind/ha) and Eurya acuminata (46 ind/ha). The IVI for adult tree were highest of Elaeocarpus sikkimensis (19.4) followed by Eurya acuminata (17.1) and Castanopsis hystrix (13.1). The species diversity (H') was highest for herbs (1.66), trees (1.54) and shrubs (1.19). Raunkiaer's life form assessment showed phanerophytes as the largest life forms (44.85%) followed by Chamaephytes (32.35%) and Geophytes (14.70%) indicating the prevalence of a phanerophytic phytoclimate in Fambong lho wildlife sanctuary (WS). The poor regeneration of oak as compared to Eurya acuminata (50.9 ind/ha), Symplocos lucida (30.9 ind/ha) indicates a high chances of change in species compositon and vegetation structure in the future.*

1. Introduction

Sikkim is a small state in the north-eastern part of India which is a repository of rich floral and faunal diversity [16]. Diversity is the result of the combination of abiotic limitations, biotic interactions and disturbances [9]. Abiotic factors such as elevation, aspect, soil texture and climate determine the physical environment and therefore heed way for the distribution of the species. The extent of biodiversity is measured typically on species level and species diversity is one of the important indices used to appraise ecosystem at different scales [1]. The diversity can be measured using various indices such as number of species per unit area (species richness), Shannon index. These indicators are used to study communities and understand the homeostatic capacity of the ecosystem to the unprecedented environmental changes [6]. Primary climate, location and condition of sites have influenced and determined biodiversity and life-forms in a particular area in the long term development of the forest ecosystems [14]. "Biological spectrum" was proposed by Raunkiaer which represented life-form distribution and the phytoclimate under which it thrives [11]. Therefore, the actual biodiversity is the result of the adaptation process of the species to its phytoclimatic condition. Oak species in general have been reported to determine the floral and faunal diversity of the forests. It has also been suggested that a shift of structure and component of an Oak forests ecosystem would likely affect the other ecosystem components as well [18]. The growth of oaks has been on the verge of decline due to hydrologic balance and its delayed response to climate [2] or due to pest infestations [5]. As Oak woodlands become denser with shade tolerant trees, plant species which are adapted to frequent disturbance and more open conditions may perish resulting in the decline in plant diversity [13]. Among the other deciduous trees oak species in general produces acorns and nuts which is a good source of food for numerous mammals and birds. The oak forests are degrading in Sikkim which has been converted into thickets, shrubs and blank areas [19]. A shift in forest tree composition from oak to other species would have serious implications for many wildlife species [13]. Therefore, to address the issue Fambong lho WS was selected as a representative site and a study was undertaken to document the species compositon, vegetation structure and its life forms under present phytoclimatic conditions.

2. Materials and Methods

2.1 Study site

Fambong lho WS was established in the year 1984 and is located in the eastern part of Sikkim covering an area of about 51.76 sq km and has its continuation with Khangchendzonga National Park in North Sikkim. The word "Fambong lho" means "orchard of "*Machilus edulis*" which is abundantly found in Sikkim Himalaya. The sanctuary is rich in biodiversity and is highly undisturbed [10]. Besides, the sanctuary is a repository of wide range of *Lithocarpus, Quercus* and *Castanopsis* species as well as several other flowering plants.

NeBIO
An international journal of environment and biodiversity
Vol. 6, No. 3, September 2015, 29 - 31
ISSN 2278-2281(Online Version) © www.nebio.info I www.neceer.org.in

Diversity and Distribution of Genus *Dioscorea* in Sikkim Himalaya, India: an application of DIVA-GIS

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ABSTRACT

The paper presents a study on diversity and distribution pattern of *Dioscorea* species in Sikkim Himalaya. *Dioscorea* is monocotyledonous tuber crop native to South East Asia. Twelve *Dioscorea* species have been collected from 105 different sites from Sikkim and adjoining Darjeeling district of West Bengal. Detailed altitudinal wise distribution mapping of species indicated its relationship with annual rainfall along different altitudinal gradients. DIVA GIS software version 7.5 was used to produce a species richness map with 0.1 x 0.1º grid cell and a circular neighbourhood of 0.1º diameter. The highest richness grid was found to have seven species while the highest diversity value was found to be 1.525 – 2.00.

KEYWORDS
Circular neighbourhood, Dioscoreaceae, Diversity index, Global Positioning System, Species Richness, worldclim

Introduction

Sikkim Himalaya (27º 4′ 46′′ to 28º 7′ 48′′ N and 88º 58′ 00′′ to 88º 55′ 25′′ E) is defined by the great drainage region of river Teesta which constitutes the hills of Sikkim and Darjeeling district of West Bengal and together covers an area of about 10,245 sq. kms habouring 4000 different species of flowering plants (Rai & Sundriyal 1997). The genus *Dioscorea* L. is the largest genus in the family Dioscoreaceae and includes more than 95% species. The family occupies a basal position among all the extant monocotyledons and was well diversified in parts of southern world at the end of Cretaceous period with early spread appearing *via* Antarctic. The genus consist about 600 species and 70 sections and is mainly distributed in Southeast Asia, Africa, Central America, South America and other tropical or subtropical regions of the world (Saikia et al 2011). Grierson (1991) described 13 *Dioscorea* species from Bhutan. In Flora of China (Gu Cuizh et al. 2007) 52 species of *Dioscorea* have been described out of which 7 species is found in Sikkim (Hajra et al. 1996) *viz. D. deltoidea* Wall. ex Kunth., *D. esculanta* (Loureiro) Burkill., *D. bulbifera* L., *D. kamonensis* Kunth., *D. pentaphylla* L., *D. alata* L., and *D. glabra* Roxb. In India the genus is represented by 50 different species mostly distributed in the west, east and northeast region of the country (Prain & Burkill 1936). So far only 13 species (seven from Sikkim) of both edible and non-edible species of *Dioscorea* have been reported from Sikkim and adjoining Darjeeling district of West Bengal (Samanta 2006). The species of *Dioscorea viz. D. pentaphylla* L, *D. bulbifera* L. and *D. deltoidea* Wall. ex Kunth. is considered medicinally important and hence the directorate of *Cinchona* and other medicinal plants situated in Mungpoo, Darjeeling district has been doing commercial cultivation of *Dioscorea* species with approximately 6000 acres, along with other medicinal and aromatic plants like *Cinchona*, Ipecac, *Citronella*, *Rauvolfia* etc. Hence this paper deals with diversity and distribution of genus *Dioscorea* in Sikkim Himalaya and its distribution is correlated with rainfall and topography of the region.

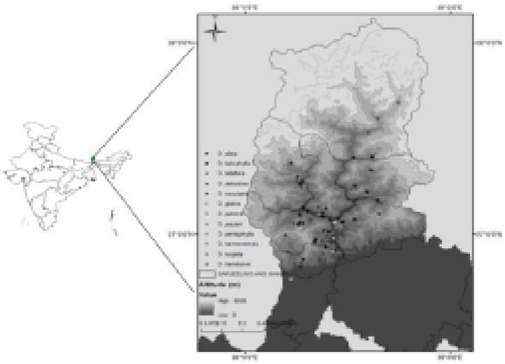

Figure 1. Distribution of *Dioscorea* species in different altitudinal zones.

Materials and methods

Species of *Dioscorea* were collected from different places of Sikkim and Darjeeling district of West Bengal during the year 2013 to 2015. The geographic coordinate of *Dioscorea* species was recorded from different places using Garmin GPS (Global Positioning System) with an accuracy of 10-20m. DIVAS-GIS software version 7.5.0 (www.divasgis.org) was used to obtain the precipitation figures for each species by querying our geographic localities with the worldclim database (Hijmans et al. 2005). The precipitation date was extracted using the option extract values by point option in DIVA-GIS software. The altitudinal grid was also downloaded from