Studies on Distribution Prediction through Ecological Niche Modeling and Propagation of Two Threatened Species *(Paris polyphylla* Smith. and *Vanda bicolor* Griff.)

By

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DECLARATION

I, **Zubenthung P. Kikon** bearing Ph. D. Registration number **538/2013 dated 18. 06. 2013,** hereby, declare that the subject matter of my Thesis entitled "**Studies on Distribution Prediction through Ecological Niche Modeling and Propagation of two Threatened Species *(Paris polyphylla* Smith. and *Vanda bicolor* Griff.)"** is the record of the work done by me, and that the contents of this Thesis did not form the basis for the award of any degree to me or to anybody else to the best of my knowledge. The Thesis has not been submitted by me for any other research degree in any other University.

This Thesis is being submitted to the Nagaland University for the degree of Doctor of Philosophy in Botany.

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Introduction

Planet earth with its unique environmental setup supports millions of life forms on earth and these assemblages of life forms sums up to form the global biodiversity. These life forms takes and contributes with each other and with their environment which is indispensable for their survival and in maintaining biological balance and removal of any component in this complex network will disturb the existing biological balance. The main emphasis of any biological diversity studies is thus focused on conservation and protection of all nature's components. The understanding of this variability of life form is not new dating back much older than the term 'Biodiversity' itself which was first conceived by Rosen (1985). Human society since time immemorial depends on resources from biological diversity for their basic necessities, these bio-resources has served as a backbone in shaping human civilization and plant kingdom has served a crucial role.

The application and usage of plants has been indispensable to men since the early ages and this necessitated application of plants for numerous end services to mankind has in some ways triggered the innovative minds of men and thus, progress and development for mankind. This has subjected plant diversity to enormous pressure and plants species are threatened and

becomes extinct with the myriad benefit to mankind that depends on plant diversity. The Living Planet Report (2014) highlights the dramatic decline in the world's biodiversity by using a global Living Planet Index which tracks 9,000 populations of more than 2,600 species. The global Index reveals that an almost 30 percent decrease in species diversity since 1970, with the highest in the tropics where there has been a 60% decline in less than 40 years (Living Planet Report, 2014). Some bioregions has higher levels of biodiversity with more endemic species, but remains under constant threat from humans activities, these regions are categorized under biodiversity hotspots. For a region to be categorized under hotspots, a region must meet two strict criteria: it must contain at least 0.5% or 1,500 species of vascular plants as endemics, and it has to have lost at least 70% of its primary vegetation (Meyers *et al.*, 2000).

Biodiversity hotspots are earth's biologically richest and most endangered terrestrial eco-regions (Meyers, 1988). There are 34 mega biodiversity hotspots in the world with India featuring four of this mega biodiversity hotspots *viz.*, Andaman and Nicobar, Himalaya, Indo-Burma region (including North Eastern region) and Western Ghats. The biodiversity hotspots holds high numbers of endemic species, yet their combined area of remaining habitat covers only 2.3% of the earth's land surface and earth's hotspots faces extreme threats and already 70% of its natural vegetation has been lost. Over 50% of the world plant species are endemic to the 34 biodiversity hotspots (www.conservation.org). North-East India which is a part of Indo-Burma is featured under the hotspots and houses a diverse vegetations type such as tropical forest, sub-tropical forest, grasslands, swamps, temperate forest, alpine forest and mixed deciduous forest. This region of India holds a rich diversity of medicinal, ornamental, endemic and other economically important species with more than 8,000 out of 15,000 species (in India) of flowering plants found in the North Eastern region, which includes 40 species of gymnosperms, 500 species of pteridophytes, 825 species of orchids, 80 species of

rhododendrons, 60 species of bamboo, and 25 species of canes (Chakraborty *et al.*, 2012). This region is viewed as an area under high threat as the indigenous people of the region are mostly dependent on forest product for provision of food, fuel, shelter and building materials. Thus a guided utilization of the limited available resources to stop global biodiversity loss, shouldered on pragmatically structured conservation planning methods, through biodiversity hotspots analysis and categorization of threat status, based on replaceability and vulnerability of species is a major challenge the world faces today.

Ecological Niche Modelling (ENM)

Environmental/Ecological niche Modelling (ENM), also known as '*Species Distribution Model' (SDM), Predictive Habitat Distribution Model,* and *Climate Envelope Model'* refers to the process of using computer algorithms to predict the distribution of species in geographic space on the basis of a mathematical representation of their known distribution in environmental space (realized ecological niche). Ecological niche modelling is based on the 'Hutchinsonian Niche Concept' who defined Niche as 'Hyper volume (or n-dimensional) space of resources (e.g., light, nutrients, structure, etc.) available to (and specifically used by) organisms'. The Hutchinsonian niche views niche as an n-dimensional hyper volume, where the dimensions are environmental conditions and the resources that define the requirements of an individual or a species to practice '*its*' way of life. The niche concept was popularized by the Zoologist G. Evelyn Hutchinson in 1957. An organism free of interference from other species could use the full range of conditions (biotic and abiotic) and resources in which it could survive and reproduce which is called its fundamental niche. However, as a result of Ecological niche Pressure from, and interactions with, other organisms (i.e. inter-specific competition) species are usually forced to occupy a niche that is narrower than this, and to which they are mostly highly adapted, this is termed as the realized niche. The environment is in most cases represented by climate data (such as temperature,

and precipitation), but other variables such as soil type, water depth, and land cover can also be used. These models allow for interpolating between a limited number of species occurrence and they are used in several research areas in conservation biology, ecology and evolution. The extent to which such modelled data reflect real world species distributions will depend on a number of factors, including the nature, complexity, and accuracy of the models used and the quality of the available environmental data layers; the availability of sufficient and reliable species distribution data as model input; and the influence of various factors such as barriers to dispersal, geological history or biotic interactions, that increase the difference between the realized niche and the fundamental niche. The fundamental ecological niche of a species is a critical determinant of its distribution; as such, it is defined in multidimensional ecological space (Mac Arthur, 1972). Several distinct interpretations of ecological niches exist but the most relevant to the present contribution was given by Grinnell (1917), who focused on the conjunction of ecological conditions within which a species is able to maintain populations without immigration. Hutchinson (1959) provided the valuable distinction between the fundamental niche, which is the range of theoretical possibilities, and the realized niche (that part which is actually occupied, given interactions with other species such as competition). Although it can be argued that only the realized niche is observable in nature, by examining species across their entire geographic distributions, species distributional possibilities can be observed against varied community backgrounds, and thus a view of the fundamental ecological niche can be assembled (Peterson and Cohoon, 1999). Understanding the Niche concept is an important issue and hence a clear picture on this issue must be addressed. In ecology, a niche is a term describing the relational position of a species or population in its ecosystem to each other; a shorthand definition of niche is how an organism makes a living. The ecological niche describes how an organism or population responds to the distribution of resources and competitors (e.g., by growing when resources are abundant, and

when predators, parasites and pathogens are scarce) and how it in turn alters those same factors (e.g., limiting access to resources by other organisms, acting as a food source for predators and a consumer of prey). Thus Ecological niche is a term for the position of a species within an ecosystem, describing both the range of conditions necessary for persistence of the species, and its ecological role in the ecosystem. This century-old concept has undergone several substantial transformations, but still represents a major heuristic tool for our understanding of nature. Niches of distinct, even closely related species tend to differ at least in some aspects as inter-specific competition minimizes their overlap to some extent, we can predict niche evolution from the knowledge of the environment and the trade-offs affecting possibilities of resource utilization of individual species. It is useful, however, to distinguish three main approaches to the niche. The first approach emphasizes environmental conditions necessary for a species presence and maintenance of its population, the second approach stresses the functional role of species within ecosystems, and the third one a dynamic position of species within a local community, shaped by species' biotic and abiotic requirements and by coexistence with other species. The emphasis on the diversity of ecological communities and inter specific competition within them in the second half of 20^{th} century has lead to the formalization of niche concept, and an emphasis on the properties of the niches which enable species to co-exist within a habitat. George Evelyn Hutchinson postulated that niche is a 'hyper volume' in multidimensional ecological space, determined by species requirements to reproduce and survive. Each dimension in the niche space represents an environmental variable potentially or actually important for species persistence. These variables are both abiotic and biotic, and can be represented by simple physical quantities as temperature, light intensity or humidity, but also more sophisticated quantities such as soil texture, ruggedness of the terrain, vegetation complexity or various measures of resource characteristics. This could be viewed simply as a formulation of original Grinnellian niche,

i.e. the exact descriptions of a species habitat requirement. However, in the Hutchinsonian view ecological niches are dynamic, as the presence of one species constrains the presence of another species by inter-specific competition, modifying the position of species niches within the multidimensional space. This concept therefore combines the ecological requirements of the species with its functional role in the local community. The niche concepts as propounded by some famous ecologist are highlighted below.

Grinnellian Niche: The word 'Niche" is derived from the Middle French word 'Nicher', meaning to nest. The term was coined by the naturalist Joseph Grinnell in 1917, in his paper *'The niche relationships of the California Thrasher'*. The Grinnellian niche concept establishes the idea that the niche of a species is determined by the habitat in which it lives. In other words, the niche is the sum of the habitat requirements that allow a species to persist and produce offspring. The Grinnellian niche is the range of values of environmental factors that are necessary and sufficient to allow a species to carry out its life history. Under normal conditions of reproduction and dispersal, the species is expected to occupy a geographic region that is directly congruent with the distribution of its niche, and the density of the species within its geographic range is expected to be correlated with the prevalence of these conditions. A study of the Grinnellian niche may seem overly broad because, except for some aspects of population biology, it is equivalent to the study of single-species geographical ecology. This is, however, precisely what is needed to avoid models that make unrealistic a prior assumptions about factors regulating distribution and abundance. The Grinnellian approach is to observe as many states of the species in nature as possible and to infer the relative effects of possible mechanisms that may constrain its distribution (Grinnell, 1917).

Eltonian Niche: In 1927 Charles Sutherland Elton, a British ecologist, gave the first working definition of the niche concept. The Eltonian niche encompasses the idea that the niche is the role a species plays in a community, rather than a habitat. The observation of distant species

adapted to equivalent ecological roles was clearly influential to Charles Elton, who emphasized the functional roles of species. According to Elton, there is the niche of burrowing detritivores, the niche of animals specializing in cleaning ticks or other parasites, or the pollination niche. Elton's niche can apply to several species, for example "the niche filled by birds of prey which eat small mammals", this functional niche therefore refers to a species position in food webs and trophic chains, and the concept is thus especially relevant for ecosystem ecology.

Hutchinsonian Niche: The Hutchinsonian niche views niche as an n-dimensional hypervolume, where the dimensions are environmental conditions and the resources that define the requirements of an individual or a species to practice 'its" way of life. The niche concept was popularized by Hutchinson (1957). An organism free of interference from other species could use the full range of conditions (biotic and abiotic) and resources in which it could survive and reproduce which is called its fundamental niche. However, as a result of Ecological niche Pressure from, and interactions with, other organisms (i.e. inter-specific competition) species are usually forced to occupy a niche that is narrower than this, and to which they are mostly highly adapted. This is termed the realized niche.

Environmental niche modelling can be considered as a part of the discipline of biodiversity informatics. Recent developments in geographic information systems and their application to conservation biology open doors to exciting new synthetic analyses. Exploration of these possibilities, however, is limited by the quality of information available. Most biodiversity data are incomplete and characterized by biased sampling. Inferential procedures that provide robust and reliable predictions of species geographic distributions thus become critical to biodiversity analyses. In this contribution, models of species ecological niches are developed using an artificial intelligence algorithm, and projected onto geography to predict species distributions. Predictive species distribution models are

empirical models relating field observations to environmental variables based on statistically or theoretically derived response surfaces. The most common strategy for estimating the potential geographic distribution of a species is to characterize the environmental conditions that are suitable for that species. The spatial distribution of environments that are suitable for a species can then be estimated across a given study region. A wide variety of techniques have been developed for this purpose, including generalized linear models, generalized additive models, bioclimatic envelopes, habitat suitability indices, and the genetic algorithm for rule-set prediction (GARP). For example, if we are interested in the distribution of a plant that is known to thrive in wet clay soils, then simply identifying locations with clay soils and high precipitation can generate an estimate of the species distribution. However there could be number of reasons why the species may not actually occupy all suitable sites (e.g. geographic barriers that limit dispersal, competition from other species) which otherwise is expected to thrive successfully in those areas. Holistic researches in exploring applications and aspects, spanning biological realms and scientific disciplines and bringing out more understanding on its application, scope and its pros and cons can open ample scope for planning species specific conservation strategies.

Historical Account

Early approaches to ENM encompass species distributions within Geographic Information System (GIS) using simple geographic envelopes, convex hulls, and environmental matching (Nix, 1986). Many geographic applications have been developed in recent years that offer exciting new possibilities for understanding biological diversity (Scott *et al.,* 2002). Geographic Information System (GIS) make it possible to build maps of species richness and endemism, to prioritize areas for conservation based on principle such as complementarities, and to access the completeness of existing protected areas network (Peterson and Cohoon, 1999). The success of such programs and approaches however

depends critically on the quality of distributional information available, which has proven to be a weak link in the process (Krohn, 1996). The SDM as we see today emerged when the statistical methods from field-based habitats studies were linked with GIS-based environmental layers. In one of the earliest applications of this integrated approach (Ferrier *et al.,* 2002) applied Generalized Linear Model (GLMs, logistic regression) to predict the distribution of the Rufous Scrub-Bird using known locality records for the species, and remotely mapped and modelled environmental variables. Contemporary SDMs combine concepts from ecological and natural history traditions with more recent developments in statistics and information technology. However the ecological roots of SDMs started in those early studies that described biological patterns in terms of their relationship with geographical and/or environmental gradients (Murray, 1866; Schimper, 1903; Grinnell, 1904). Moreover, research that has highlighted on the individuals responses of species to their environment (MacArthur, 1972; Whittaker, 1973) provided the strong conceptual argument for modelling individual species rather than communities. Modern quantitative modelling and mapping of species distributions emerged when two parallel streams of research activity converged. On the one hand field-based ecological studies of species-habitat associations, at first reliant largely on linear multiple regression and discriminate function analyses (Capen, 1981; Stauffer, 2002), benefitted from new regression methods that provided coherent treatments for the error distributions of presence-absence and abundance data. Generalized Linear Models (GLMs) enabled pioneering regression-based SDMs that has much more sophistication and realism than was possible earlier (Austin *et al.,* 1990). The key structural features of GLMs (non-normal error distributions, additive terms, non-linear fitted functions) continue to be useful and are part of many current methods including resource selection funct ions/RSF (Manly *et al.,* 2002) Maximum Entropy Models/MAXENT (Phillips *et al.,* 2006). At the same time, rapid methodological advances in physical geography provided new data

and information systems. New data allowed robust and detailed preparation of digital models of the Earth's surface elevation, interpolation of climatic parameters, and remote sensing of surface conditions in both marine and terrestrial environments. These greatly enhanced, SDM capabilities by providing estimates of environmental conditions across entire landscapes, including retrospection at surveyed areas. Many works has been done on Ecological Niche but very few species has been studied in detail in terms of their dynamic response to environmental change, and static distribution often remains the only approach for studying the possible consequences of a changing environment on species distribution (Woodward and Cramer, 1996).

Methods and Techniques

A large number of algorithms have been used in species distribution modelling. They can be classified as 'profile', 'regression', and 'machine learning' methods. Profile methods only consider 'presence' data, not absence or background data. Regression and machine learning methods use both presence and absence or background data. The distinction between regression and machine learning methods is not very distinct, but it is perhaps still useful as way to classify models. Another distinction that one can make is between presence-only and presence-absence models. Profile methods are always presence-only, other methods can be either, depending if they are used with survey-absence or with pseudo-absence/background data. An entirely different class of models consists of models that primarily use the geographic location of known occurrences or only uses these known location, and do not rely on the values of predictor variables at these locations. General approaches on the Working Methodologies of some algorithms are discussed below.

Profile Methods

***Bioclim*:** Bioclim is a classic 'climate-envelope-model'. Although it generally does not perform as good as some other modelling methods (Elith *et al.*, 2006), particularly in the context of climate change (Hijmans and Graham, 2006), it is still used, among other reasons because the algorithm is easy to understand and thus useful in teaching species distribution modelling. The BioClim algorithm computes the similarity of a location by comparing the values of environmental variables at any location to a percentile distribution of the values at known locations of occurrence ('training sites'). The closer to the 50th percentile (the median), the more suitable the location is. The tails of the distribution are not distinguished, that is, 10 percentile is treated as equivalent to 90 percentile.

Domain: The Domain algorithm (Carpenter *et al.*, 1993) has been extensively used for species distribution modelling. It did not perform very well in a model comparison (Elith *et al.*, 2006) and very poorly when assessing climate change effects (Hijmans and Graham, 2006). The Domain algorithm computes the Gower distance between environmental variables at any location and those at any of the known locations of occurrence (training sites). If averaging is enabled, the value stored is the average of the "n" nearest cells. Analyses are generally conducted with n=1, but larger values can be useful in reducing effects of outlier training points

***Mahalanobis*:** The 'Mmahal' function implements a species distribution model based on the Mahalanobis distance (Mahalanobis, 1936). Mahalanobis distance takes into account the correlations of the variables in the data set, and it is not dependent on the scale of measurements.

Regression Models

***Generalized Linear Models*:** A generalized linear model (GLM) is a generalization of ordinary least squares regression. Models are fit using maximum likelihood and by allowing

the linear model to be related to the response variable via a link function and by allowing the magnitude of the variance of each measurement to be a function of its predicted value. Depending on how a GLM is specified it can be equivalent to (multiple) linear regression, logistic regression or Poisson regression (Guisan *et al.,* 2002) gave an overview of the use of GLM in species distribution modelling.

Generalized Additive Models (GAM): Generalized additive models (Hastie and Tibshirani, 1990; 2009; Wood, 2006) are an extension to GLMs. In GAMs, the linear predictor is the sum of smoothing functions. This makes GAMs very flexible and they can fit very complex functions. It also makes them very similar to machine learning methods.

Machine Learning Methods

***MaxEnt*:** MaxEnt (Maximum Entropy) (Phillips *et al.,* 2004; 2006) is the most widely used SDM algorithm. Elith *et al.* (2011) provides an explanation of the algorithm (software) geared towards ecologists. MaxEnt is available as a Stand-alone Java program. It uses presence only data for model development and performs well even with small sample size.

***Boosted Regression trees*:** Boosted Regression Trees (BRT) is, unfortunately, known by a large number of different names. It was developed by Friedman (2001), who referred to it as a "Gradient Boosting Machine" (GBM). It is also known as "Gradient Boost", "Stochastic Gradient Boosting", and "Gradient Tree Boosting".

***Random Forest*:** The Random Forest (Breiman, 2001) method is an extension of Classification and regression trees (CAR) (Breiman *et al.,* 1984).The function random Forest can take a formula or, in two separate arguments, a data Frame with the predictor variables, and a vector with the response. If the response variable is a factor (categorical), Random Forest will do classification, otherwise it will do regression.

The procedure for developing environment model may differ in the algorithm and the data set that is used to develop the model and many workers has given different approaches using different Algorithms e.g., **BIOCLIM (**Beaumont and Hughes, 2002); **DOMAIN** (Carpenter *et al.,* 1993); **Classification and Regression Tree Analysis (CTA/CART/RTA)** (Iverson and Prasad, 1998); **Logistic Regression/Binomial GLM** (Guisan and Theurillat, 2005); Generalized Additive Models (GAM) (Araujo and Luoto, 2007); **ANN**-Artificial Linear Models (Pearson and Dawson., 2003); Genetic Algorithm for Rule set Prediction (**GARP)** (Meyer *et al.,* 2006; Peterson *et al.,* 2006; Peterson, 2001); Generalized Linear Models **(GLM**); **GAM, CTA, ANN** (Araújo *et al.,* 2005)**; MaxEnt and GARP** (Cayuela *et al.,* 2009; Adhikari *et al.,* 2012; Haredasht *et al.,* 2013); **MaxEnt** (Phillips *et al.,* 2006; Phillips and Dudik, 2008; Kumar and Stohlgren, 2009; Gonzalez *et al.,* 2011; Kulhanek *et al.,* 2011). However, the basic steps for model development follow a relatively similar pathway in all the earlier works that has been studied. The procedure of SDM building has different component which are described below (Guisan and Zimmerman, 2000).

1. Conceptualization

***a. Theory and data acquisition*:** Define an up-to-date conceptual model of the system to be stimulated based on sound ecological and well defined objectives (Austin, 2002), setting multiple working hypotheses like pseudo equilibrium (Guisan and Theurillat, 2000) working out the available and missing data and the relevance of environmental predictors for the target species and the scale (Thuiller *et al.,* 2005) identifying a feasible sampling strategy for collecting new data (Hirzal and Guisan, 2002) or for complementing existing sets, and choosing the appropriate spatio-sampling resolution and geographic extend for the study.

***b. Defining modeling methods*:** Identify the best suited algorithm to carry out modeling works and the most suited modeling methods(s) for variable response and identifying both the

framework and the statistics needed for evaluating the predictive accuracy of the model (Pearce and Ferrier, 2000a). In current practice, due to the lack of knowledge of the target organism or of the study area and related data, very few decisions are made at the start of the study. For instance the choice of the right data resolution might depend on the size of the species home range and the way the species uses to resources in the landscape. The choice of the geographical extent might also depend on a prior knowledge of environmental gradients in the study area (Austin and Gaywood, 1994).

2. Data Preparation

Data can be from primary or secondary sources and occurrence data can be obtained from primary source mainly through filed surveys as well as recording the geographic co-ordinate of the area where the target species has been observed using Global Positioning Systems (GPS). Occurrence data can also be obtained as secondary source from published literature (research papers and monographs), herbarium and also from online biodiversity databases such as Global biodiversity Information Facility (GBIF), HerpNet, MaNIS, OBIS, ORNIS, REMIB and SINGER. Climate and environment data used in modeling are basically of two types:

I. ***Continuous data*** where the variables are measurements done in a continuous scale e.g., interpolated raster data on temperature, precipitation and remote sensing data such as NVDI.

II. ***Categorical data*** representing discrete data characteristics e.g., land use, forest types, vegetation types, soil types etc. The most commonly used data set in Niche Modeling is that of the Worldclim which available is for free in Worldclim website (http://www.worldclim.org/) developed by Hijmans *et al.,* (2005) and can be downloaded in different resolutions besides this environment data can be downloaded at different website sources like http://www.cru.uea.ac.uk/, http://pmip.lsce.ipsl.fr/, http://glovis.usgs.gov/, etc. these datasets can be further modified for applicability to the logarithms.

3. Model Fitting

Identifying the dependent or independent variables and the data frame that holds this variable**.**

4. Model Evaluation and Assessment of Model Applicability

Developing a model and making a prediction can be easy comparing to when assessing how good the model is and whether it can be used for the specified purpose. Most models have different measures that can help assess the goodness of fit. Most modelers rely on cross-validation method where as model is developed with separate training and testing data through random sampling from a single set, and fewer cases where training and test data are from different sources and predefined. Different methods of evaluating models can be used to evaluate the quality of a prediction, and many of the evaluating methods based on presence-absence or presence-only data are threshold dependent. Currently the discrimination of the ability of the Species Distribution Model is mainly assessed using two measures the Kappa statistics, and the Area Under Curve (**AUC**) of a Receiver Operating Characteristics (**ROC**) plot (Fielding and Bell, 1997). The kappa coefficient measures the correct classification rate of correctly classified presences and absences after the probability of chance agreement has been removed. Landis and Koch (1997) proposed a scale to describe the degree of concordance based on Kappa: **0.8-1**(almost perfect), **0.61-0.81**(substantial), **0.41-0.6**(moderate), **0.21-0.4** (fair), **0.00-0.2**(fail). An alternative measure of accuracy is the AUC of the ROC plot; AUC relates relative proportions of correctly classified (true positive proportion) and incorrectly classified (false negative proportion) cells over a wide and continuous range of threshold levels. This makes it a threshold of independent measure (Pearce and Ferrier, 2000b) an approximate guide for classifying the accuracy of the Area Under Curve (AUC) was proposed by Swets (1998): **0.90-1.00** (Excellent), **0.80-0.90** (Good),

0.**70-0.80** (Fair), **0.60-0.70** (Fair), **0.50-0.60** (Fail). AUC value of less than 0.5 indicates that the model tends to predict presence in area where species are in face absent. In some case species range shift projections (Beaumont and Hughes, 2002) were made without attempts to validate the predictive accuracy of models being presented.

ENM and Fields of Application

Ecological Niche has found its application in many fields like setting up conservation priorities (Meyer *et al.,* 2006; Adhikari *et al.,* 2012); biodiversity (Sinclair *et al.,* 2010); plant biodiversity (Heikkinen *et al.,* 2006); Land use (Hijmans *et al.,* 2005); climate change (Brereton *et al.,* 1995; Pearson and Dawson, 2003); medicinal plants (Gaikwad *et al.,* 2011); endemic and endangered species (Giriraj *et al.,* 2008; Babar *et al.,* 2012); invasive species (Adhikari *et al.,* 2012); Human Disease (Adhikari *et al.,* 2009; Haredasht *et al.,* 2013; Hay *et al.,* 2013). A new approach of merging ENM and biotechnology is also coming up where biotechnological tools like tissue culture techniques can be used for mass multiplication of selected plant species and their re-introduction in areas predicted suitable through ENM, which will ensure survival of the introduced plantlets. Niche models which is rather new in India can be a very useful tool in areas like Nagaland where the indigenous practice of farming has put immense pressure on the forest Bio-Diversity. The local practices like Jhuming and burning of forest destroys a huge chunk of well forested areas every year, these anthropogenic activities has limited species expansion disturbing the meta-population and dynamic structure of species. Rapidly changing environmental conditions, manmade or natural has forced species to an entirely new set of climatic conditions causing a vast decline in the niche of species. This could lead to situations where species being wiped out before their documentation and their usages identified, as such selected species can be spatially identified and areas prioritization and conservation can be stressed in locations with high prevalence of the target species.

Predictive distribution models in the recent years have gained popularity with the advancement in information technology. However, the question of their applicability in designing specific conservation strategies and the level of authenticity still has some questions to be addressed. Sources of uncertainty in bioclimatic models have been the talk for modelers in the statistical and ecological literature. Factors associated with uncertainties in model building includes, decisions associated with model designing, Collinearity, choice of variable and quality control. All modelling techniques has their advantages and disadvantages and the choice of modelling approaches needs to take into consideration a range of factors on the extend of ecological knowledge, existing population data, spatial and temporal scale as well as goal of the modelling works (Pearson and Dawson, 2003). Recent works has highlighted the need for research aiming to develop approaches that integrate factors such as land cover, biotic interactions and dispersal mechanisms into species climate models. A study to address these challenges have been increasing but still is in need of substantial research, and developing hybrid-models that brings together the best of correlative with the best of mechanistic and theoretical models is one of the most important challenges for modellers (Araujo *et al.,* 2006) as well as developing models that can correlate the dynamic response of the target species to the challenging environmental conditions. Because of the various sources of uncertainty it may be conceptually inadequate to use the projections of bioclimatic models as the face value for making predictions of future events. However, when limitations of models are understood, we should be in a better position to make the best use of their results (Whittaker *et al.,* 2005). The fact that the physical world with all its dynamicity makes it practically impossible to bring out a model as accurate as one expects as “An error not resisted is approved” (Chapman, 2005). But addressing uncertainties in all possible aspects will help produce models and more empirical evidence needs to be gathered to reinforce the confidence of bioclimatic models and their predictions (Araujo *et al.,* 2005) and by providing

repeated evidence we should be in a position to use their outputs with confidence for specific purposes.

Plant Propagation

Propagation of plants was the basic occupation of humans when men started farming and domestication of food crops, which initiated stable communities and ancient civilizations. Since the origin of agriculture the domestication of plants particularly food plants became popular and the need for more food and food security is the possible reason that has lead to discovery of diverse plant propagation procedures and techniques. Early approaches to plant propagation was the conventional method of selecting the best fruit and using it as the planting material for the coming season and this method is responsible for the slow genetic modification and improvement of our present day crop plants. At its nascent stage plant propagation was confined with the selection of those plants with better qualities but slowly plant breeders develop different techniques of asexual and vegetative propagation methods. Some popular conventional plant propagation techniques includes air or ground layering, division, drafting and bud grafting, micropropagation, stolons or runners, storage organs such as bulbs, corms, tubers and rhizomes, striking or cuttings, twin-scaling, offsets etc. These conventional techniques of plant propagation remains popular to present day plant breeders.

With the discovery of the fundamental laws of inheritance by Mendel's and the discovery of DNA structure by Watson and crick allows precise plant modification going deep to genetic level and thus the new era biotechnology where genetically modified crop plants and plants with enhanced ability like severe stress survival, high productivity and disease resistance was established. With the advent of biotechnology and discovery of different plant growth hormones the approach to plant propagation also change with biotechnological tools like tissue culture and micro-propagation techniques allowing mass

multiplication of plants using any parts of the plant. The present research works explores the potentials of both conventional macro-propagation and micro-propagation to bring out the best suitable propagation procedures for two plant species *viz. Vanda bicolor* (orchid) and *Paris polyphylla* (medicinal plant).

***Macro Propagation*:** Propagation of plants from parts of the parent plant by using techniques like air or ground layering, division, drafting and bud grafting, micropropagation, stolons or runners, storage organs such as bulbs, corms, tubers and rhizomes, striking or cuttings, twin-scaling, offsets etc comes under macro-propagation. Macro-propagation or vegetative propagation techniques for plant propagation has seen a big leap when the American Society for Horticultural Science was formed in 1903, with new techniques of vegetative propagation becoming popular among plant breeders and cultivars. In horticulture plants produced from sexual reproduction are rarely used and are confined mainly in research studies for conserving the richness of the gene pool and developing new varieties. The new plantlets from the sexual reproduction may carry unpredictable characters, which may reduce production and may even affect the gene pool of the plants. The practice of vegetative propagation of fruit trees goes back to ancient times. Chinese, Greeks and Romans had been using grafting techniques since pre-classical times sharing these techniques to the world. Propagation techniques like cuttings using different plant parts like stems, roots and leaves; layering methods like air layering, tip layering, trench layering and serpentine layering; Grafting techniques like whip grafting, saddle grafting, side grafting, splice grafting, cleft crafting, veneer grafting etc, with sound knowledge on scion and understock callus growth; natural vegetative propagation through runners, stolons, rhizomes, bulbs, rhizomes etc was an important propagation techniques and becoming a popular means of plant propagation (Preece, 2003).

***Micropropagation*:** Those techniques using biotechnological tool like tissue culture to mass multiply important plant species comes under micro-propagation. Plant cell/tissue culture,

also referred to as *in vitro*, axenic, or sterile culture, has become an important tool in both researches as well as in commercial application. Plant Tissue Culture (PTC) consists of a set of different techniques and procedures to coax and manipulate cells to let them grow. Some of the different PTC techniques are callus, suspension cultures, protoplasts, anther and ovule cultures, somatic embryos, and meristem culture (Dodds and Roberts, 1995). Depending on species employed and the kind of response that is desired, almost any part of a plant can be used as explants, type of explants commonly used are leaf portions, isolated meristems, hypocotyls, or root segments among others. Micropropagation or Plant tissue culture has witness significant changes and improvements since the time when Gottlieb Haberlandt address to the German Academy of Science in 1902 on his experiments on the culture of single photosynthetic cells and establishing the cell as having potentialities of being elementary organism, in his experiment the cells increased in size, accumulated starch but failed to divide. Thus, Haberlandt's experiment failed as the cultured plant cells could grow, divide and develop into embryo and then to whole plant. This potential of a cell is known as totipotency. Subsequently Hannig (1904) use embryogenic tissue, he excised nearly mature embryos from seeds crucifers and successfully grew them on mineral salts and sugar solution to maturity. In 1908, Simon regenerated callus, buds and roots from Poplar stem segments and established the basis for callus culture. Kotte (1922a, 1922b), a student of Haberlandt, and Robbins succeeded in culturing isolated root tips. This approach, of using explants with meristematic cells, led to the successful and indefinite culture of tomato root tips using yeast extract (White, 1934). Approaches to PTC was revolutionized with the discovery of the first plant growth regulator (PGR) by Went (1928), indoleacetic acid (IAA) a naturally occurring member of a class of PGRs called 'auxins'. The first true plant tissue cultures was done on cambial tissue of *Acer pseudoplatanus* using agar-solidified medium of Knop's solution, glucose, and cysteine hydrochloride supplemented with auxin enhances the proliferation of

cambial cultures (Gautheret, 1934). Further, Murashige and Skoog (1962) developed a new medium where some salts were 25 times that of Knop's solution, Murashige and Skoog medium is currently the most widely used medium. In particular, the level of nitrate and ammonia were very high and with increase in micronutrients. This medium allowed for a further success in the number of plant species that could be cultured, many of them using only a defined medium consisting of macro- and micronutrients, a carbon source, reduced nitrogen, B vitamins, and growth regulators (Gamborg *et al.,* 1976). The nutrition of PTC requires a culture medium which can provide both organic compounds and inorganic salts and in addition to a carbon source and plant growth regulators (George, 1993). The successful application of PTC is profoundly influenced by the nature of the culture medium used. The main difference among media could be the salt concentration levels mainly the amount and quality of the nitrogen source (Murashige and Skoog, 1962). It is also important to note that some culture media's components are not only nutrients, but some of them can have a very deep influence either in the growth of the cultures, or in the differentiation process (Wetherell and Dougall, 1976). With continued improvisation and expansion the application of PTC has been used in all types of plants including ornamental plants like Orchids.

The origin of orchid micropropagation/tissue culture are connected with the history of plant tissue culture, phytohormones and other areas of plant physiology. The first in vitro orchid propagation was carried out by Rotor (1949) using *Phaelanopsis* flower stalk; later Thomale (1956) was the first to culture an orchid shoot tip and Morel (1960) on *Cymbidium* shoot tip culture and reporting the successful development of protocorm-like bodies (PLBs) which could be sub-cultured and later different workers reported orchid tissue culture using stems, flower buds, flowers, floral segments and other reproductive organs.

Advancement in tissue culture and related researches has gained momentum in the past decades and development of protocols for *in vitro* regeneration of plants have been developed

for many plant species including medicinal (Choi *et al.,* 1998; Chaturvedi, 2007; Kaur and Bhutani, 2009; Arenmongla and Deb, 2012); ornamental (Deb and Pongener, 2013); rare and threatened species (Temjensangba and Deb, 2005; Deb and Temjensangba 2007a; Deb and Pongener, 2009). *In vitro* propagation of plant species through different explants source like seeds (Katiyar *et al.,* 1987; Deb and Temjensangba 2007b; Deb and Sungkumlong, 2009); foliar segments (Vij and Pathak 1990; Temjensangba and Deb 2005; Deb and Temjensangba 2007a; Deb and Sungkumlong 2010), aerial roots (Vij and Sharma, 1997; Deb and Temjensangba, 2006); meristems (Morel, 1960); nodal segments (Gulati *et al.,* 1996); rhizomes (Salvi *et al.,* 2002); cotyledons (Choi *et al.,* 1998) etc. Cost effective and eco-friendly protocols for tissue culture have also been developed for reducing the price tag of tissue culture techniques and also reducing pollution from tissue culture waste (Deb and Pongener, 2013). This potentiality of plant tissue culture in mass multiplication of plantlets has opened avenues for their application in many fields like food security, economic uses and even in plant conservation strategies for economically important plants and those plant species under threat.

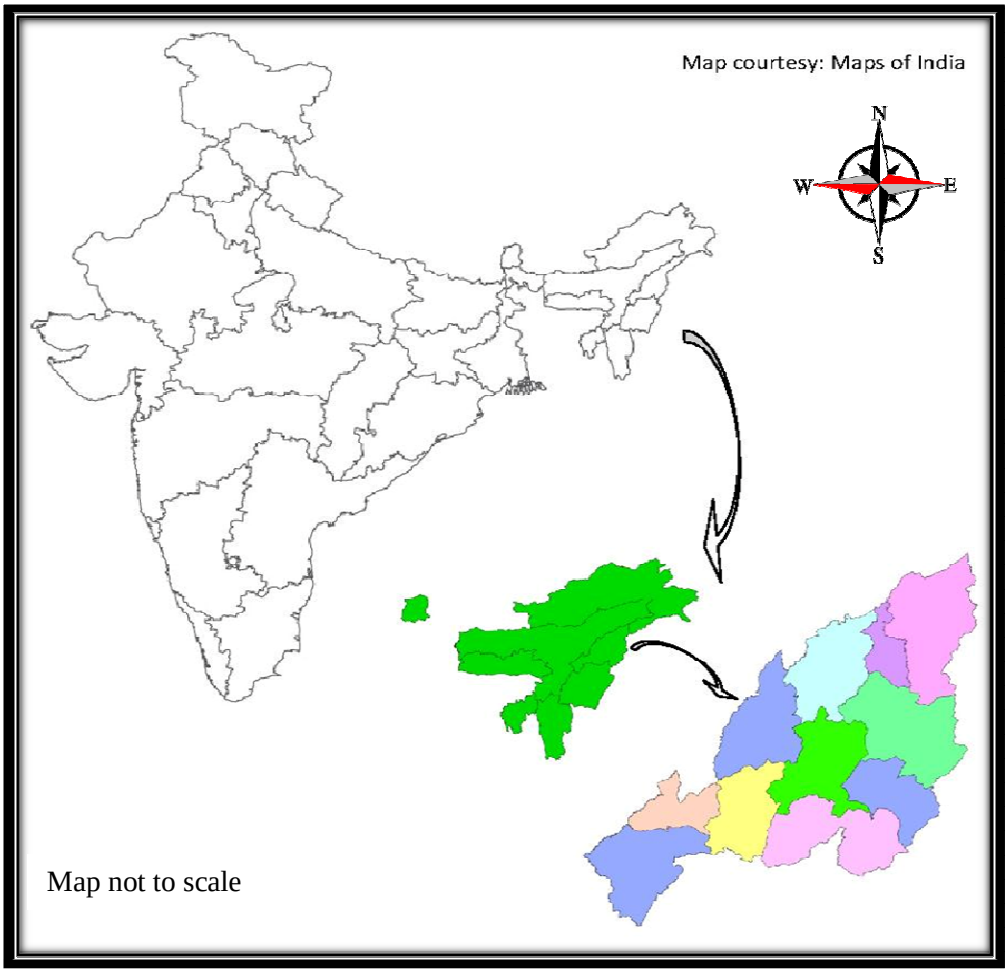

Figure – 1.1

Figure - 1.1: Map of India showing the study site (Nagaland).

Genesis of the Present Study

Prevailing conventional agricultural practices coupled with rampant exploitation of natural resources like 'Jhum', deforestation, excessive collection of economically important plant species has reduced the plant population in the region. Environmental conservation policies and laws are not being governed effectively by the policy makers, where more emphasis is on traditional practice of resources utilization. Thus the need of modern GIS technologies and tissue culture technique for species recovery and re-introduction can be a promising solution. The present study explores and brings out ample scope for propagation,

large scale production and prioritizing areas for conservation of two economically important plant species *viz*. *Paris polyphylla*, and *Vanda bicolor*. Their propagation in nature through seeds and other asexual means are highly compromised by various anthropogenic activities and inability of the seeds to germinate in case of orchids. Propagation of these plant species through *in vitro* and *ex vitro* techniques for their regeneration and mass multiplication will provide means for their rapid multiplication and applying GIS and Environment modelling technology for their introduction to substantiate their re-introduction in areas where the environment conditions are apt for their growth and multiplication in nature. The present study was focused on the development of protocols for propagation and developing climate models for prediction of occurrence, re-introduction and prioritizing areas for conservation of the two threatened plant species of North East India.

Study Site

The study was carried out in Nagaland (**Figure - 1.1**) which is a part of the Indo-Burma hotpots that is home to 2.3% of global endemic plant species (Meyers, 2000) which lies between 93^{o}20´- 95^{o}15´ East Longitude and 25^{o}31´- 27 ^{o}1´ North latitude of the equator consisting of a narrow strip of hilly area running Northeas t to Southwest which is located in the northern extension of Arakan Yoma Range borders the state of Assam to the west, Arunachal Pradesh and part of Assam to the north, Myanmar to the east and Manipur to the south, with a total geographical area of 16,579 km². The state has a forest cover of 13,044 km² (78.68%) according to Forest survey of India, (India state of Forest Report 2013) with a total forest cover loss of 274 Km^2 since 2011 report. The state falls under seven forest types *viz.* Tropical Wet Evergreen, Tropical Semi Evergreen, Tropical Moist Deciduous, Subtropical Broad Leaved Hill, subtropical pine and Montane Temperate Forests (India State of Forest Report, 2011) with an average rainfall of 1583mm (Kusre and Singh, 2012).

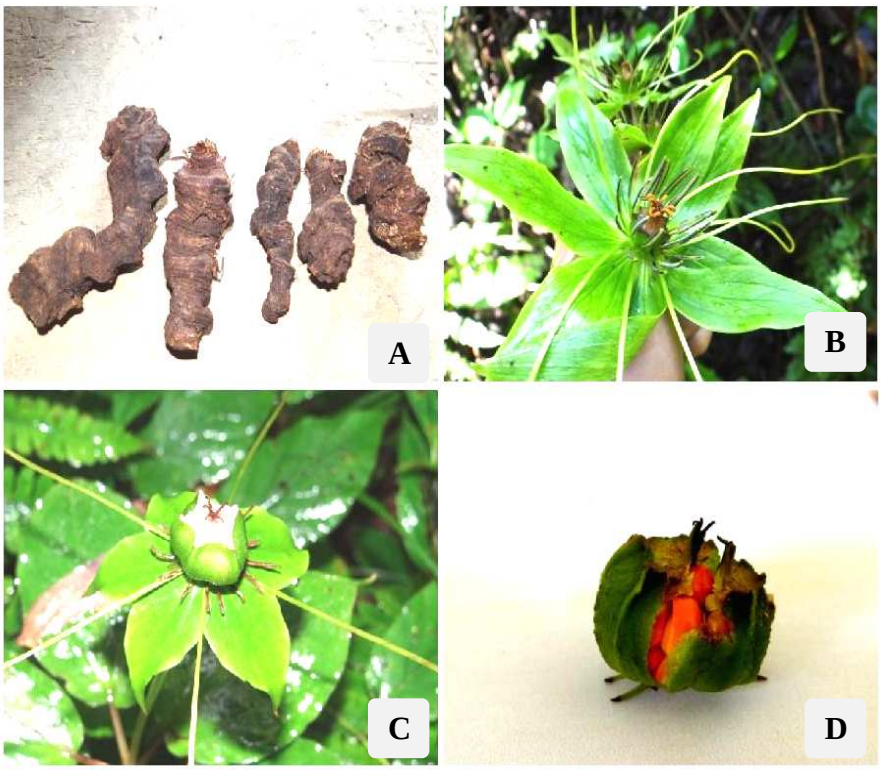

Figure – 1.2

Figure - 1.2: *Paris polyphylla* Griff. A. Rhizome, B. Inflorescence, C. Seed bearing plant and D. Matured seed pod.

Brief Description of the Target Plant Species

A. *Paris polyphylla*

Botanical name - *Paris polyphylla* Griff.

Synonym - *Diaswa polyphylla.*

Family - Melanthiaceae

Threat status - IUCN - Vulnerable (Madhu *et al., 2010*).

Habit - Perennial Herb erect herb, 15-50 cm.

Salient morphological features - Rhizome stout, creeping. Leaves 4-9 in a whorl, short-stalked, elliptic, finely acuminate, base cuneate, glabrous. Flowers solitary, terminal, short-stalked; pedicel sepals (3-) 4(-6), lanceolate, acuminate, Petals equaling number of sepals,

(*1/2*- 2/3) longer than sepals, filiform, yellowish or greenish. Stamens 10, short. Stigma lobes usually 4, re-curved at tips. Fruit globular; seeds scarlet (**Figure - 1.2 A, B, C, D**).

Distinguishing characters **-** Leaves 4-9 in a whorl short-stalked, elliptic, finely acuminate, base cuneate, glabrous. Flowers solitary, terminal, short-stalked; pedicel sepals (3-) 4(-6), lanceolate, acuminate, Petals equaling number of sepals, (*1/2*- 2/3) longer than sepals, filiform, yellowish or greenish.

Phenology (flowering and fruiting period) - Usually the flowering season is April to May and fruiting season is July.

Habitat (forest type/site condition - Broad-leaved and mixed woodlands to 3000 meters in the Himalayas. Forests, Bamboo forests, thickets, grassy or rocky slopes and stream sides, 100-3500 meters in western China (266). *Forest types* - Tropical Wet evergreen forest, tropical Semi Evergreen forest and Sub-tropical broad leaved wet hill forest.

Distribution (global/India/state/local)- East. Asia – China to the Himalayas. India (Nagaland, Manipur, Mizoram and other parts of N. E. India).

B. *Vanda bicolor* Griff.

Synonym - No synonyms recorded for this name

Family - Orchidaceae

Vernacular name - Likya (Lotha)

S**tatus** - Rare

Habit - Epiphytes

Salient morphological features- Stout erect herbs, 20-90 cm high. Leaves narrowly oblong, 15-25 * 2-3 cm; apex obliquely truncate, toothed or unequally and obtusely 2 lobed. *Inflorescence* racemose, lax, 4-6 flowered, erect or sub-erect, 10-15 cm long. *Flowers* white-purplish, mottled above, violet tinged beneath, *ca.* 5 cm across ; bracts pale brown, *ca.*4mm

long; *sepals* obovate, spathulate, obtuse, tessellate, undulated at margin 2.2 0.8 cm. *lip ca.* 2 cm long, white, violet tinged, *side lobes* oblong or suborbicular, *mid lobes* panduriform; disc smooth. *Column* whitish violet *ca*, 6mm long. *Pollinia* ovoid; *ovary* pedicelled, White, 4-5 cm long (**Figure - 1.3**).

Figure – 1.3

Figure - 1.3: *Vanda bicolor* (with flowers and green pod).

Distinguishing characters **-** *Flowers* white-purplish, mottled above, violet tinged beneath, *ca.* 5cm across; bracts pale brown, *ca.*4mm long; *sepals* obovate, spathulate, obtuse, tessellate, undulated at margin 2.2 0.8 cm. *lip ca.* 2 cm long, white, violet tinged, *side lobes* oblong or suborbicular, *mid lobes* panduriform;

Phenology (flowering fruiting period)- March - June

Habitat (forest type/site condition) - Epiphytic, up to 800 m, Forest types- Tropical Wet evergreen forest, tropical Semi Evergreen forest and Sub-tropical broad leaved wet hill forest

Distribution (global/India/state/local) - This orchid has an endemic distribution restricted to Indo-Burma regions of India Arunachal Pradesh, Assam, Nagaland, Sikkim; Bhutan, Myanmar, Nepal (Noltie, 1994. Hynniewta *et al*., 2000; Pearce and Cribb, 2002; De and Hajra, 2004).

Objectives

- **Development of climate suitability model of *Paris Polyphylla* for distribution prediction using ecological niche modeling tools.**
- **Development of climate suitability model of *Vanda bicolor* for distribution prediction using ecological niche modeling tools.**
- **Propagation of *Paris Polyphylla* Smith.**
- **Propagation of *Vanda bicolor* Griff.**
- **Field establishment of regenerates.**

Chapter -2

Distribution Prediction of *Paris polyphylla* through Climate Suitability Modelling

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With the change in the habitat scenario of biodiversity, the niche radius for many species is either rendered inhabitable for the species or is conflicted with Humans. Anthropogenic activities have destroyed the natural habitats of many plant species, pushing them to the brink of extinction. This issue unless addressed will lead to loss of immense biological wealth for Mankind. The present study gives a model for the conservation and re-introduction of selected medicinal plant species through climate suitability modelling in Nagaland, India.

Nagaland is known for its tribal population diversity with 16 major tribes and their rich culture and traditions, also has a distinct character both in terms of its social composition as well as in its developmental history. Each of the 16 tribes celebrating their festivals every month giving the tag “Land of Festivals”, the region has provided a safe abode from climatic extremities thus far and experiences mild environmental conditions offering safe and clean

habitats. With growth and development coupled with the increase in population, plant species are in constant threat as old customary practices and traditions of conservation strategies are forgotten or lost with each new generation taking over.

The present study was focused on the distribution prediction of *Paris polyphylla* belonging to family Melanthiaceae. *Paris polyphylla* is a multipurpose medicinal herb highly prized for its medicinal properties and for the purpose the species is over exploited from its natural habitat. *Paris polyphylla* has found its application as antidote, analgesic, ethnopediatrics in diarrhea, medicine for antifebrile, alexipharmic, detumescent, demulsent, haemostatic, haemopathy and many more (Shah *et al.,* 2012) . The plant has multiple applications in ancient Chinese medicine which is being practiced till now. The propagation of the plant is difficult because of the high level of seed dormancy of more than 18 months as the seeds undergo secondary dormancy, requiring two winters and one summer in natural environments (Li, 1984). Rhizome propagation is also possible but regeneration is low and takes long time to propagate thus needing an alternative, through conservation of the plant in their natural and suitable habitats. Mapping the possible potential distribution of the species for possible re-introduction is done with the help of Ecological Niche Modeling (ENM) which co-relates the climatic conditions of known areas where the species occur to predict their potential distribution.

Species distribution prediction has become a popular and important component of planning conservation strategies in recent years, and for this purpose different algorithms and modeling techniques have been developed (Guisan and Thuiller, 2005). These models employs the associations between environmental variables and known species occurrence records to identify environmental conditions within which populations can be maintained and flourish. The spatial distribution of environments that are suitable for the species can then be estimated across a target study region. This method has shown to be valuable for generating

bio-geographical information that can be used in a broad range of studies including conservation planning studies. The sum total of all climatic factors of an area determines the behavior and survival of species. Species prevalence is determined by a set of physico-climatic factors and not by one factor alone (Grinnell, 1917). Through ENM a representation of the climatic factors of known occurrence areas in mathematical form are used to describe the potential suitable areas based on the data supplied in computer algorithm. Common strategy for estimating the actual or potential geographic distribution of a species is characterizing the climatic conditions that are suitable for the species, and the identification of where these suitable environments are distributed in space. For example, if we are interested in modeling the distribution of a plant that is known to thrive in wet clay soils, then simply identifying locations with clay soils and high precipitation can generate an estimate of the species' distribution. There are other possible reasons why the species may not actually occupy all predicted suitable sites (e.g. geographic barriers that limit dispersal, competition from other species). The present study was aimed to bring out the possible suitable areas of occurrence which were subjected to different validation process besides maintaining statistical significance, by observation of plant behavior introduced to both suitable and unsuitable areas as predicted by the model to establish its validity. Comparisons of the climate factors identified by the model as a key in determining the species survival with an earlier temperature stratification experiment on seed germination of the plant species under study shows the response of the plant species at different climatic parameters and how the model effectively brings out those parameters in those areas with high prediction threshold.

Materials and Methods

Algorithms Used

- **DIVA GIS 7.5**
- **ArcGIS 10.2.2**
- **MaxEnt 3.3.3e**

Different modelling algorithms have been applied to classify the probability of species' presence (and absence) as a function of a set of environmental variables. The present study employs MaxEnt algorithm to develop the model as MaxEnt software is based on the maximum-entropy approach for species habitat modelling. This software takes a set of layers or environmental variables (such as elevation, precipitation, etc.), as well as a set of geo referenced occurrence locations as inputs, and produces a model of the range of the given species. The model for a species is determined from a set of environmental or climate layers for a set of grid cells in a landscape, together with a set of sample locations where the species has been observed. The model expresses the suitability of each grid cell as a function of the environmental variables at that grid cell. A high value of the function at a particular grid cell indicates that the grid cell is predicted to have suitable conditions for that species. The computed model is a probability distribution over all the grid cells. The distribution chosen is the one that has maximum entropy subject to some constraints: it must have the same expectation for each feature (derived from the environmental layers) as the average over sample locations (Phillips *et al.,* 2010). All other spatial analysis works like data conversion, importing/exporting, mapping and visualization was done in DIVA GIS and ArcGIS.

Input Data

Climate Data: Different types of environmental variables have been used as input in species' distribution modelling. Most common ones are climate variables like (e.g. temperature, precipitation), topography (e.g., elevation, aspect), soil type and land cover type. These environmental variables may be either *continuous data* (data that can take any value within a certain range, such as temperature or precipitation) or *categorical data* (data that are split into different categories, such as land cover type or soil type). For the present study bioclimatic variables were obtained from Worldclim at 30´´ pixel resolution, which consist of an interpolated datasets of temperature and precipitation which are of primary importance for

the plant to thrive and reproduce successfully at a particular area. Worldclim version.1 was developed by Hijmans *et al.* (2005). The climate elements considered were monthly precipitation and mean, minimum, and maximum temperature and the 19 bioclimatic variables with different climatic parameters (**Table - 2.1**).

Table - 2.1: The 19 bioclimatic variables showing the different climatic parameters

Bioclimatic No.	Climatic parameter
BIO1	Annual mean temperature
BIO2	Mean diurnal range (max temp – min temp)(monthly average)
BIO3	Isothermality (BIO1/BIO7) * 100
BIO4	Temperature seasonality (Coefficient of Variation)
BIO5	Max. temperature of warmest period
BIO6	Min. temperature of coldest period
BIO7	Temperature annual range (BIO5-BIO6)
BIO8	Mean temperature of wettest quarter
BIO9	Mean temperature of driest quarter
BIO10	Mean temperature of warmest quarter
BIO11	Mean temperature of coldest quarter
BIO12	Annual precipitation
BIO13	Precipitation of wettest period
BIO14	Precipitation of driest period
BIO15	Precipitation seasonality (Coefficient of Variation)
BIO16	Precipitation of wettest quarter
BIO17	Precipitation of driest quarter
BIO18	Precipitation of warmest quarter
BIO19	Precipitation of coldest quarter

Table - 2.2: 12 Occurrence points that were used to develop the model

	Admin 1	District	Area	Species	Altitude (m ASL)	Longitude	Latitude
1	Manipur	Ukhrul	Ukhrul	*Paris polyphylla*	1779	94° 21′67″	25° 05′1″
2	Meghalaya	Shillong	Upper Shillong	*P. polyphylla*	1731	91° 48′80″	25° 32′0″
3	Nagaland	Wokha	Mt.tiyi	*P. polyphylla*	1557	94° 6′.050″	26 ° 6′.323″
4	Nagaland	Mokokchung	Longkhum	*P. polyphylla*	1405	94° 7′.035″	26° 5′.988″
5	Nagaland	Tuensang	Pangsa	*P. polyphylla*	1906	95° 7′19.2″	26°15′46.7″
6	Nagaland	Wokha	Mt.Tiyi	*P. polyphylla*	1325	94° 18′24.2″	26° 07′00.3″
7	Nagaland	Wokha	Mt.tiyi	*P. polyphylla*	1140	94°17′23.0″	26° 07′27.3″
8	Nagaland	Zunheboto	Aizuto	*P. polyphylla*	1545	94° 30′57″	26° 09′07.1″
9	Nagaland	Kohima	Khonoma	*P. polyphylla*	1880	94° 0′36.3″	25° 37′47.7″
10	Nagaland	Kohima	Khonoma	*P. polyphylla*	1459	94° 1′40.5″	25° 38′59.3″
11	Nagaland	Kohima	Dzulekhe	*P. polyphylla*	1670	93° 6′13.4″	25° 37′06.1″
12	Nagaland	Kohima	Dzulekhe	*P. polyphylla*	1814	93° 8′49.9″	25° 38′10.6″

Occurrence Data*:** Twelve GPS points of the plant species (**Table - 2.2**), geo-referenced during primary ground surveys using GPS (eTrex 10) were used as occurrence points, all the occurrence points were subjected to quality test with respect to and their positional accuracy was ascertained through Google earth, duplicates were identified and removed thus maintaining only one point within 1×1 Km^2 to avoid sampling bias which would otherwise favour the climatic of those sites where sampling is highly concentrated. The geo referenced points are converted to Decimal Degrees (DD) format using MS. Excel with a precision of four decimals from Degree Minute Seconds (DMS) format using the common formula; **Decimal degrees = [(Degrees (°) + Minutes (′) / 60 + Seconds (″) / 3600)] * H. Where *H = 1 when the coordinate is in the Eastern (E) or Northern (N) Hemisphere H = -1 when the coordinate is in the Western (W) or Southern (S) Hemisphere

As the number of presence points in below 20 (*i.e.* 12) 1.5x InterQuartile Range (1.5 IQR) method of identifying outliers is applied to check for outliers based on climate data developed from the environmental data obtained from Worldclim Website at 30″. All climate data are cross checked for resolution accuracy and corrected to 30″ pixel resolution.

For MaxEnt the coordinates are fed in longitude and latitude as MaxEnt can handle most coordinate systems provided that the Comma-Separated Value (.csv) file coordinates match the coordinate system of the spatial data layers.

***Model Calibration*:** All modelling works was carried out using MAXENT Version 3.3.3K as the present work was based on presence points only and has low sample size and MAXENT can efficiently handle small sample size as described by Philips *et al.* (2006). All visualization was done in DIVA GIS 7.5.0, and all mapping works was carried out using ARC GIS 9.3. Two models were developed using Jackknife method (Pearson *et al.*,

2007). This method is called *k*-fold partitioning. In this method the data are split into *k* parts of roughly equal size ($k > 2$) and each part is used as a test set with the other *k*-1 sets used for model calibration. Thus, if we select $k = 4$ then four models will be calibrated and each model tested against the excluded test data. Validation statistics are then reported as the mean and range from the set of *k* tests (Fielding and Bell, 1997). An extreme form of *k*-fold partitioning, with *k* equal to the number of occurrence localities, is recommended for use with very low sample sizes (e.g., < 20; Pearson *et al*., 2007). This method is also called 'leave-one-out' since each occurrence locality is excluded from model calibration during one partition. The first model was developed using all the 19 bioclimatic variables whereas the second model was developed using only monthly temperature and precipitation data (**Table – 2.3, 4**).

***Model Validation and Authentication*:** For validating model robustness, 20 and 12 replicated model runs was executed for the first and second prediction model with a threshold rule of 10 percentile training presence and employed cross validation technique for dividing the samples into replicate folds and using as test data all other parameters were kept at default following Adhikari *et al.,* (2012) (Table 2.3, 4). The AUC was graded according to Thuiller *et al.,* (2005). The distribution potential of the model was classified into very low, low, medium, high and very high.

To authenticate the model intensive field surveys was carried out in the different prediction threshold areas the presence and absence of the target species was noted with respect to the prediction map developed.

Table - 2.3: Relative contributions of the environmental variables to the MAXENT Model-1 using the 19 Bioclimatic variables

Variable	Percent contribution	Permutation importance
BIO6	44.1	32.7
BIO4	17.4	0.1
BIO18	9.5	2.1
BIO7	6.4	13.6
BIO5	4.4	0
BIO14	4.1	30.6
BIO16	3.8	0.2
Alt	3.4	0.8
BIO13	3.3	0.2
BIO12	1.3	0
BIO17	0.8	10.3
BIO19	0.6	7.1
BIO8	0.4	1.1
BIO3	0.2	1
BIO10	0.1	0
BIO15	0.1	0

Table - 2.4: Relative contributions of the environmental variables to the MAXENT Model using only temperature and precipitation data (Model-2)

Variable	Percent contribution	Permutation importance
tmin12	19.6	0
tmin1	16.8	46.8
prec12	16.4	13.5
prec3	10.9	22.8
prec9	9.7	0
tmax7	8.1	0
tmax1	6.2	3.1
prec7	3.8	0.1
prec8	1.2	0.5
prec11	1.1	12.7
prec6	1.0	0.1

Results

Model 1: The model calibration for the first model (**Figure - 2.1**) gives a test AUC of 0.983 and AUC train of 0.99 with a standard deviation of 0.039. The AUC thus ranges from 0.5 for models that are no better than random to 1.0 for models with perfect predictive ability. The AUC test is derived from the Receiver Operating Characteristic (ROC) Curve. The ROC curve thus describes the relationship between the proportion of observed presences correctly predicted (sensitivity) and the proportion of observed absences incorrectly predicted (1-specificity). Thus, an AUC value of 0.7 means the probability is 0.7 that a record selected at random from the set of presences will have a predicted value greater than a record selected at random from the set of absences (Fielding and Bell, 1997; Pearce and Ferrier, 2000).

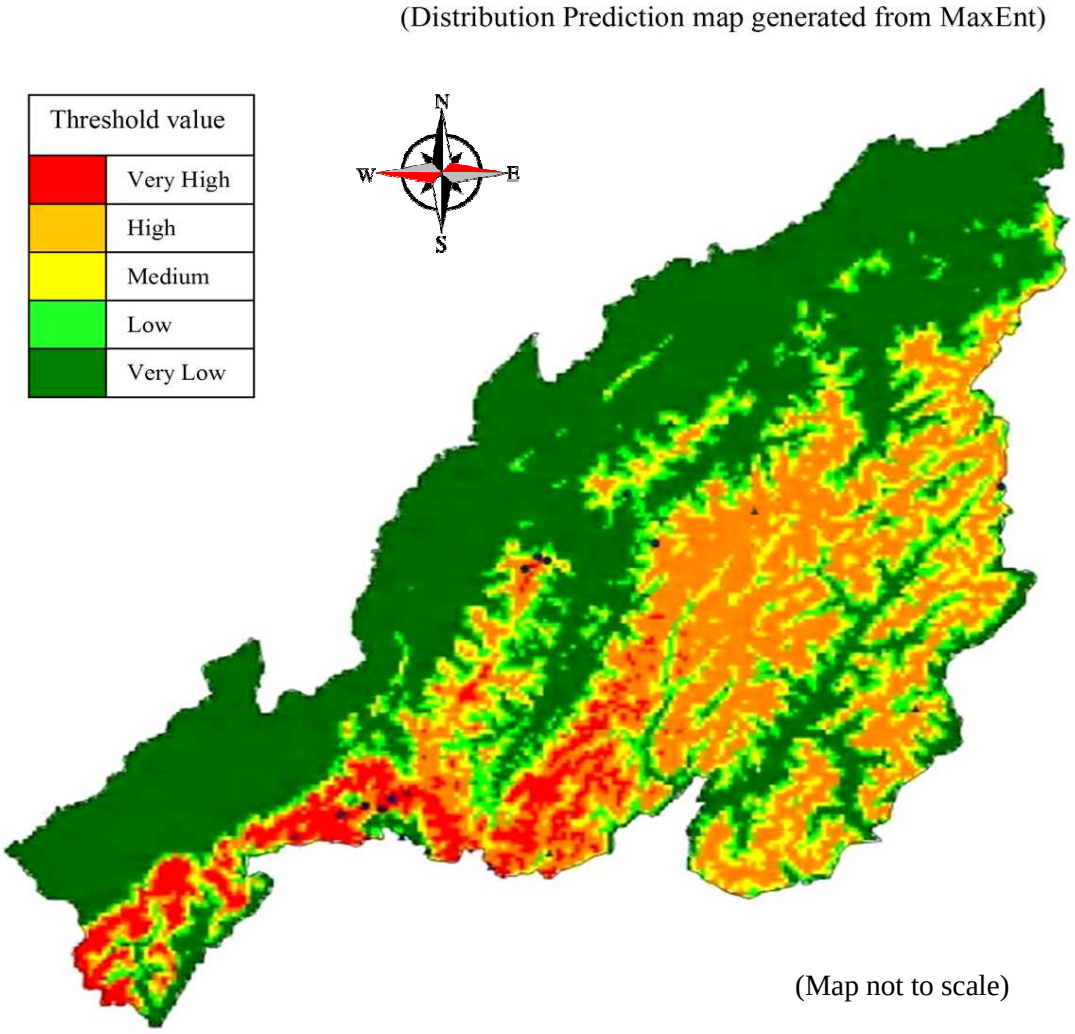


Figure – 2.1

Figure - 2.1: Model - 1. Distribution prediction map of *Paris polyphylla* (using 19 Bioclimatic).

Table - 2.3 gives estimates of relative contributions of the environmental variables to the MaxEnt model showed that BIO6 (Minimum Temperature of Coldest Month) contributed the maximum (**Figure - 2.1**) 44.1% followed by BIO4 (Temperature seasonality, standard deviation x 100) and BIO18 (precipitation of warmest quarter) contributing 17.4 and 9.5% respectively.

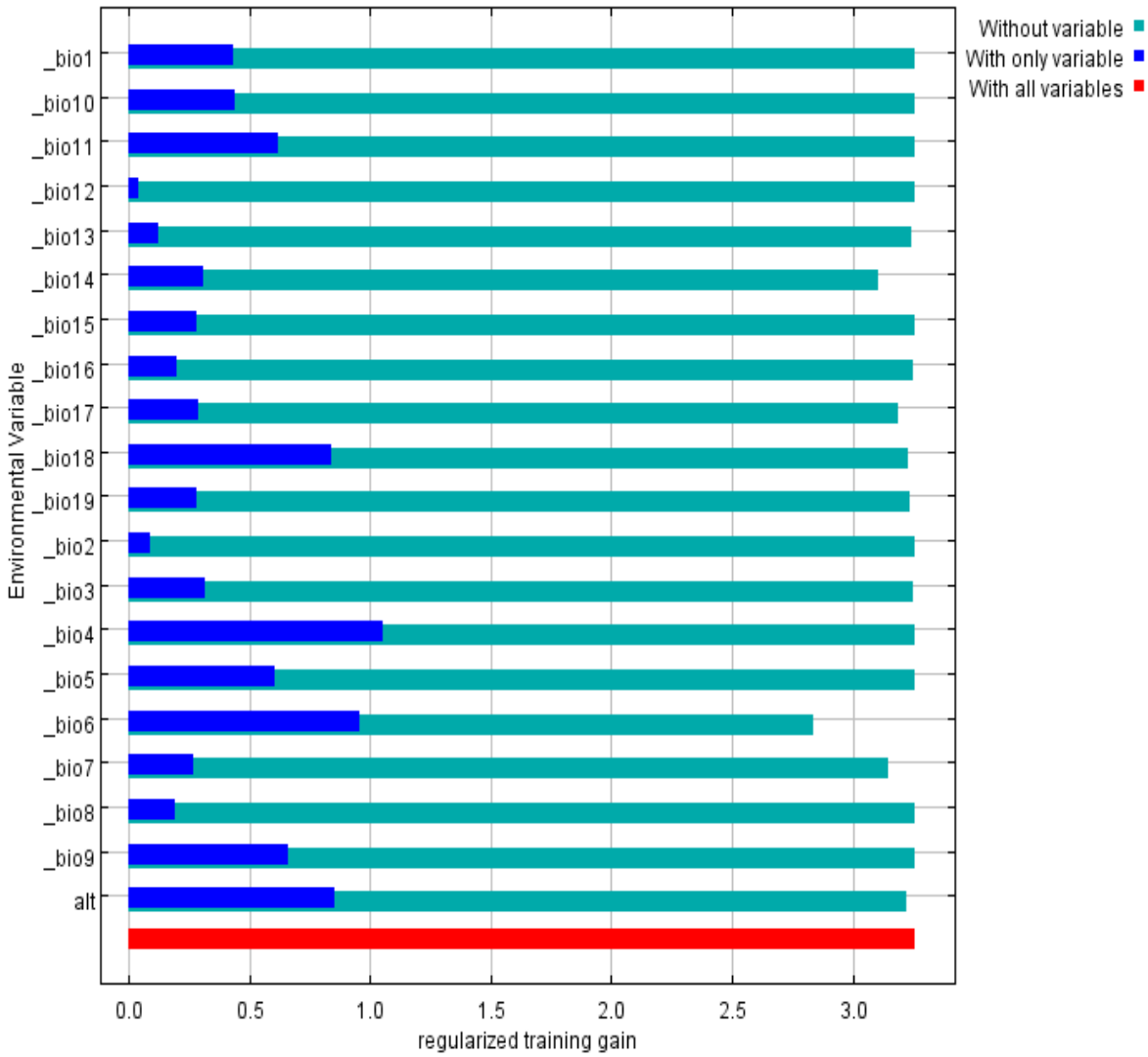


Figure – 2.2

Figure - 2.2: Model – 1. Result for the Jackknife test of variable importance.

Table - 2.5: Prediction values for the different districts surveyed

District	Prediction Values
Kohima	0.85
Kohima	0.64
Kohima	0.59
Kohima	0.4
Kohima	0.82
Peren	0.92
Mon	0.56
Tuensang	0.73
Phek	0.77

Table - 2.6: Monthly climatic variables and the corresponding months with their contribution in the prediction model

Variable	Corresponding Months	Percent contribution
tmin12	Minimum Temperature in the month of December	19.6
tmin1	Minimum Temperature in the month of January	16.8
prec12	Precipitation in the month of December	16.4

Table - 2.7: Natural temperature stratification phenomenon that was occurring in the predicted high suitable areas and the Bioclimatic variable that is determining the distribution model

Months of year with average temperature	Bioclimatic variable identified
1st Year December-January (0-5^{o}C)	BIO6 (Minimum Temperature of Coldest Month)
1st Year May-September (20-26^{o}C)	BIO18 (precipitation of warmest quarter)
2nd Year December-January (0-5^{o}C)	BIO6 (Minimum Temperature of Coldest Month)
2nd Year May-September (20-2^{o}6C)	BIO18(precipitation of warmest quarter)

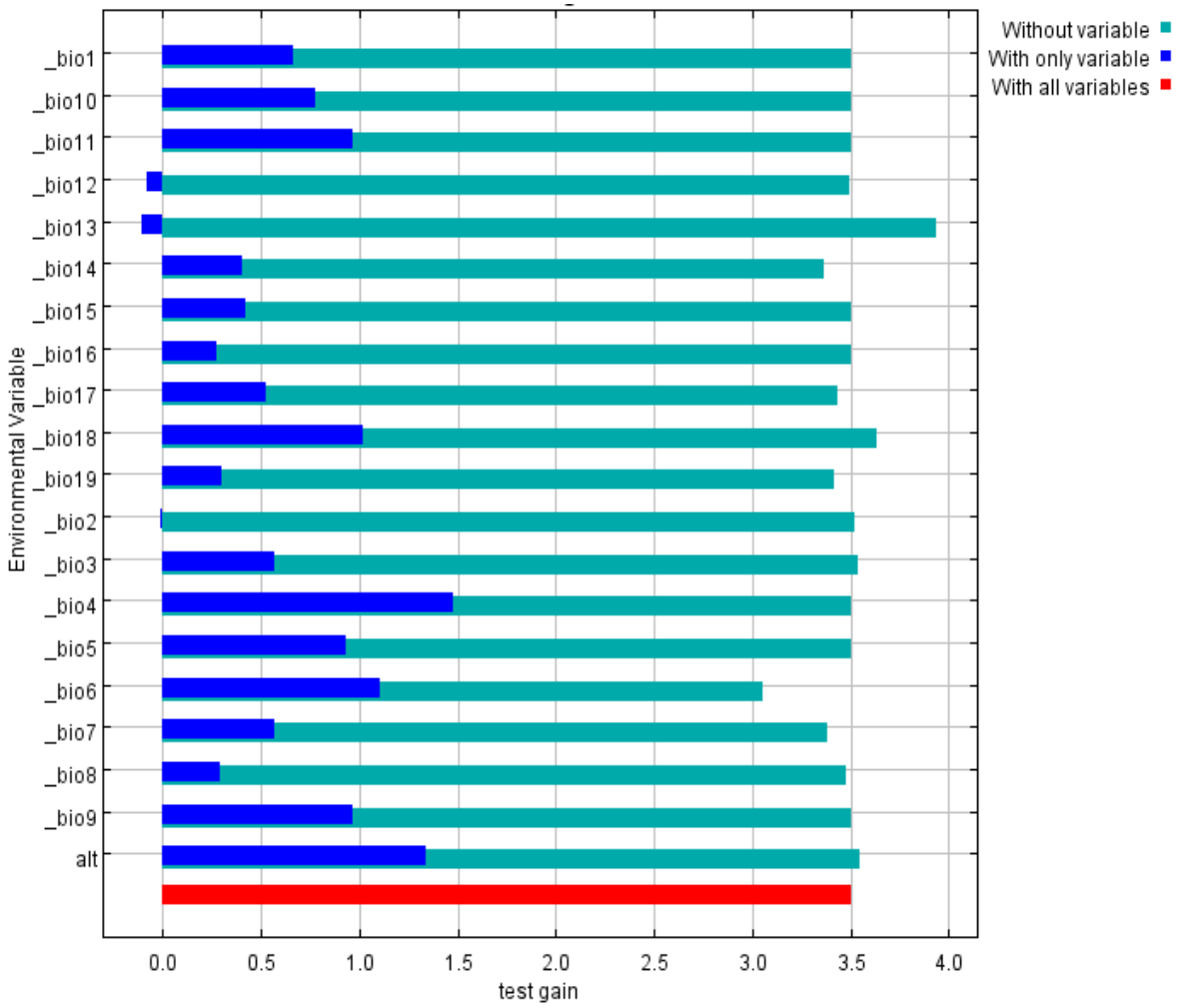


Figure – 2.3

Figure - 2.3: Model -1. Result for the Jackknife test using test gain.

MaxEnt Jackknife test of variable importance (**Figure - 2.2, Table 2.4**) shows that BIO4 (temperature seasonality) allows a reasonably good fit to the training data. The environmental variable with highest gain when used in isolation is BIO4, which therefore appears to have the most useful information by itself in the model. The environmental variable that decreases the gain the most when it is omitted is BIO6 (Minimum Temperature of Coldest Month), which therefore appears to have the most information that isn't present in the other variables (Table 2.6, 7).

Same Jackknife test, using test gain instead of training gain also shows that BIO4 and BIO6 as an important variable in test data gain, the test gain plot shows that a model made only with BIO12 (annual precipitation) and BIO13 (precipitation of wettest month) results in a negative test gain (**Figure - 2.3**). The model thus is slightly below a null model (i.e., a uniform distribution) for predicting the distribution of occurrences set aside for testing. This means the annual precipitation and precipitation of wettest month values are not the useful as predictor variables.

Jackknife test using AUC on test data (**Figure - 2.4**), the AUC plot shows that BIO4 (temperature seasonality) is the most effective single variable for predicting the distribution of the occurrence data that was left aside for testing, when the predictive performance is measured using AUC, though it was hardly used by the model built using all variables and the relative importance of BIO4 also increases in the test gain plot. These results shows that temperature variables are playing an important role for MaxEnt to obtain a good fit to the training data with the seasonal temperature variable defining better results on the set-aside test data (Most useful variable as predictor).

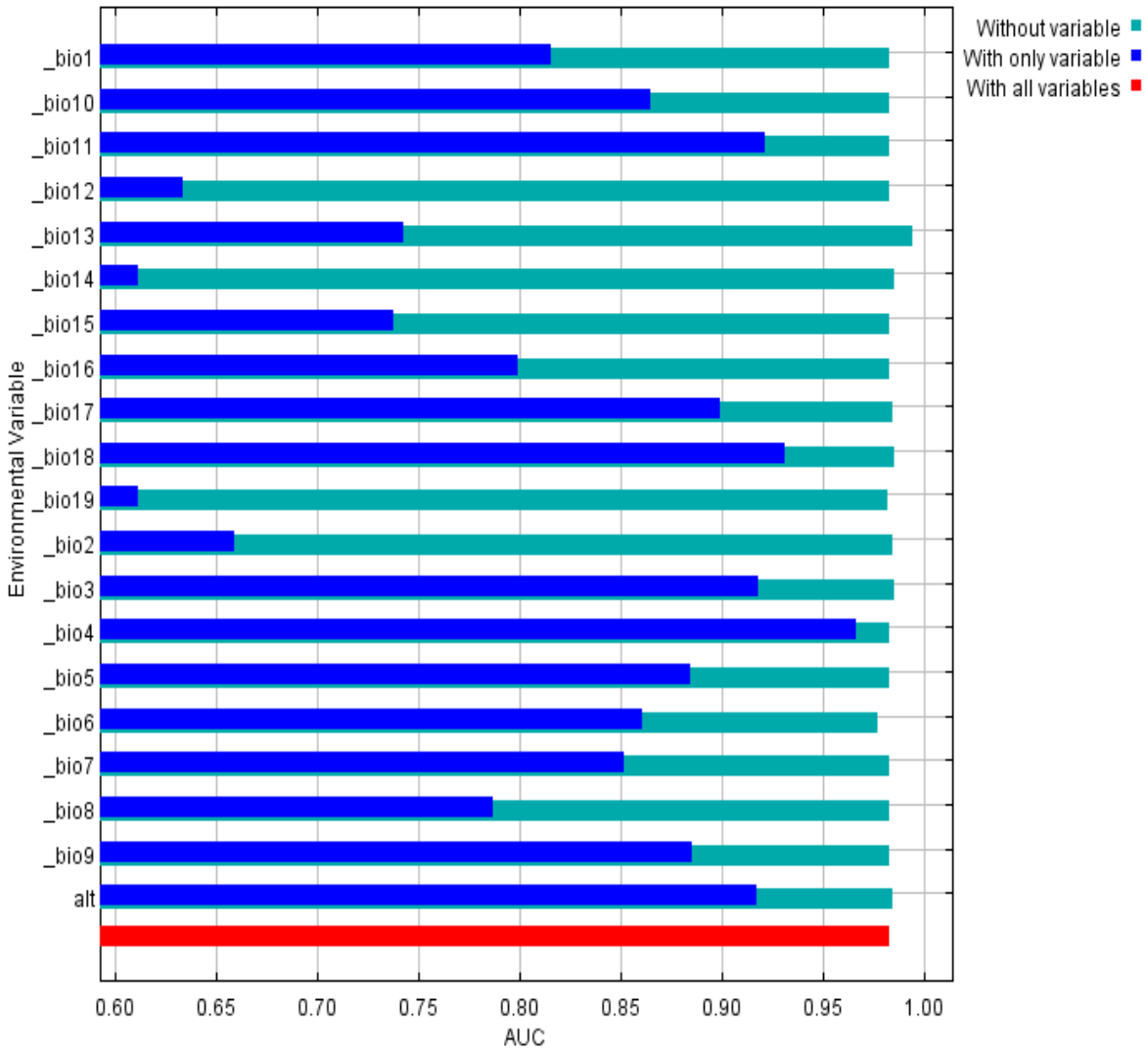


Figure – 2.4

Figure -2.4: Model-1. Jackknife test, using AUC on test data.

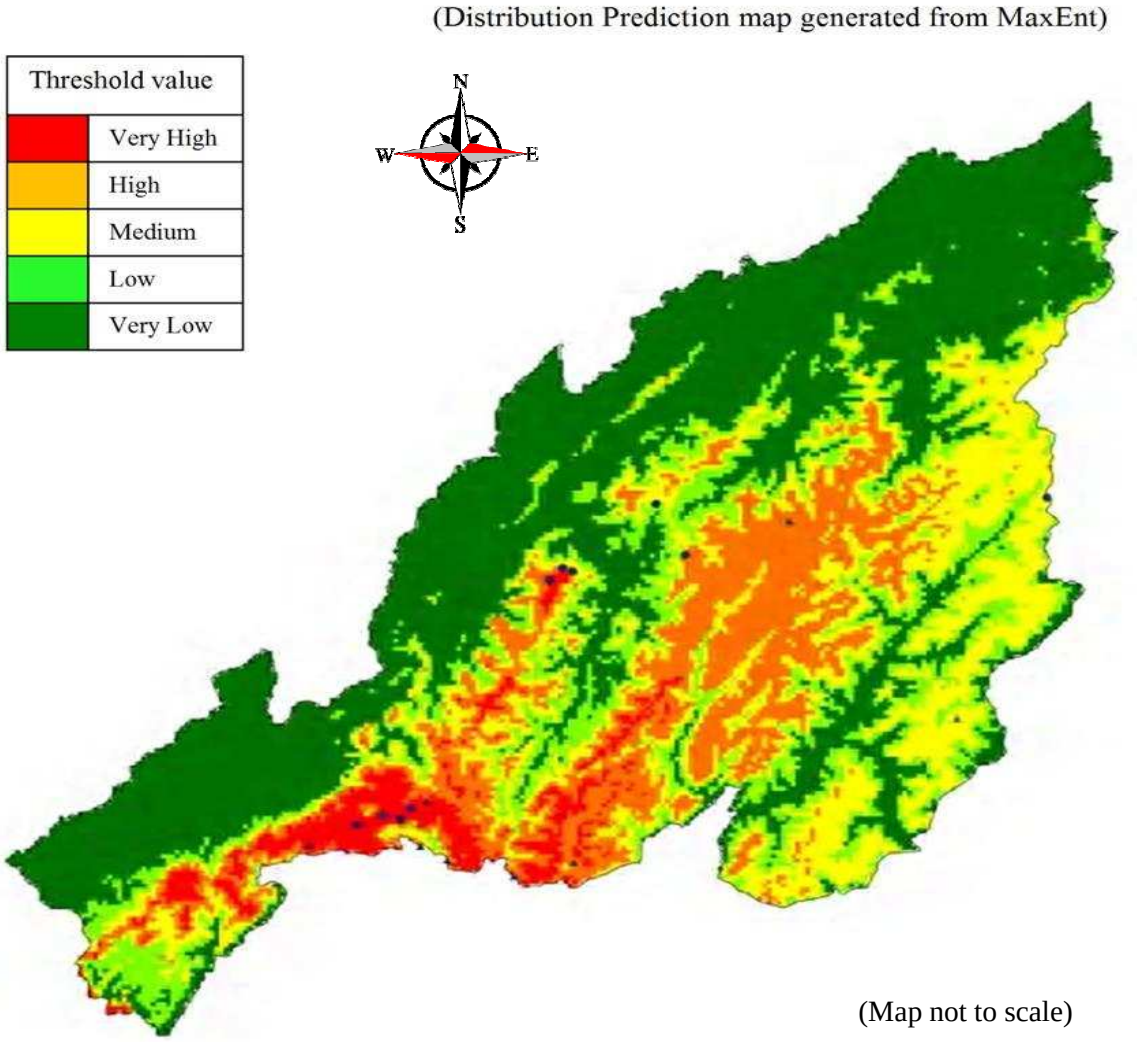


Figure – 2.5

Figure – 2.5: Model-2. Distribution prediction map of *Paris polyphylla* (using temperature and precipitation).

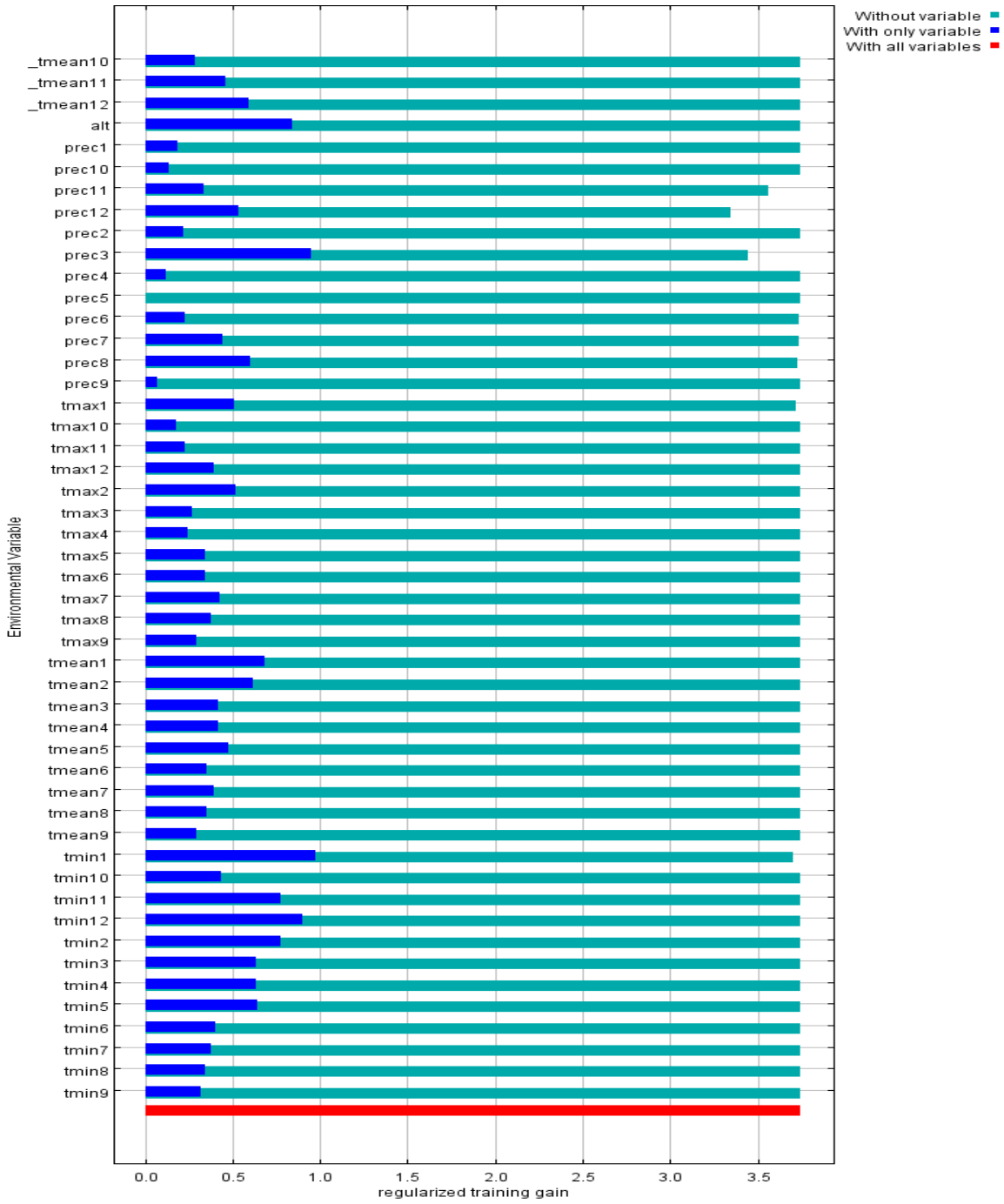


Figure – 2.6

Figure – 2.6: Model - 2. Result for the Jackknife test of variable importance.

Model 2: The second model (**Figure - 2.5**) was trained using the 12 presence points and only monthly temperature and precipitation data was used as climate data to find out the impact of the different monthly climatic variable. Calibration of model was done as before but 12 replicated runs gives a better result among the other models with different number of replicated runs. 12 replicated runs gives a test AUC of 0.974, AUC train of 0.99 and the standard deviation is 0.075. Table - 2.3 gives estimates of relative contributions of the environmental variables to the MaxEnt model shows that Tmin12 (temperature in the month of December) and Tmin1 (temperature in the month of December) giving the most relative contribution in predicting the probable habitat distribution.

MaxEnt Jackknife test of variable importance (**Figure - 2.6**) shows Tmin12 (temperature in the month of December) giving a reasonably good fit to the training data. The environmental variable with highest gain when used in isolation is BIO4, which therefore appears to have the most useful information by itself in the model. The environmental variable that decreases the gain the most when it is omitted is prec12 (precipitation in the month of December), which therefore appears to have the most information that is not present in the other variables.

Same jackknife test, using test gain instead of training gain (**Figure -2.7**) also shows that minimum temperature in the month of January and December along with mean temperature in the month of January as an important variable in test gain, the test gain plot also shows that a model made only precipitation data of the month of May, June and October results in a negative test gain (**Figure - 2.5**). The model thus is below a null model (i.e., a uniform distribution) for predicting the distribution of occurrences set aside for testing and the variables are not the useful as predictor.

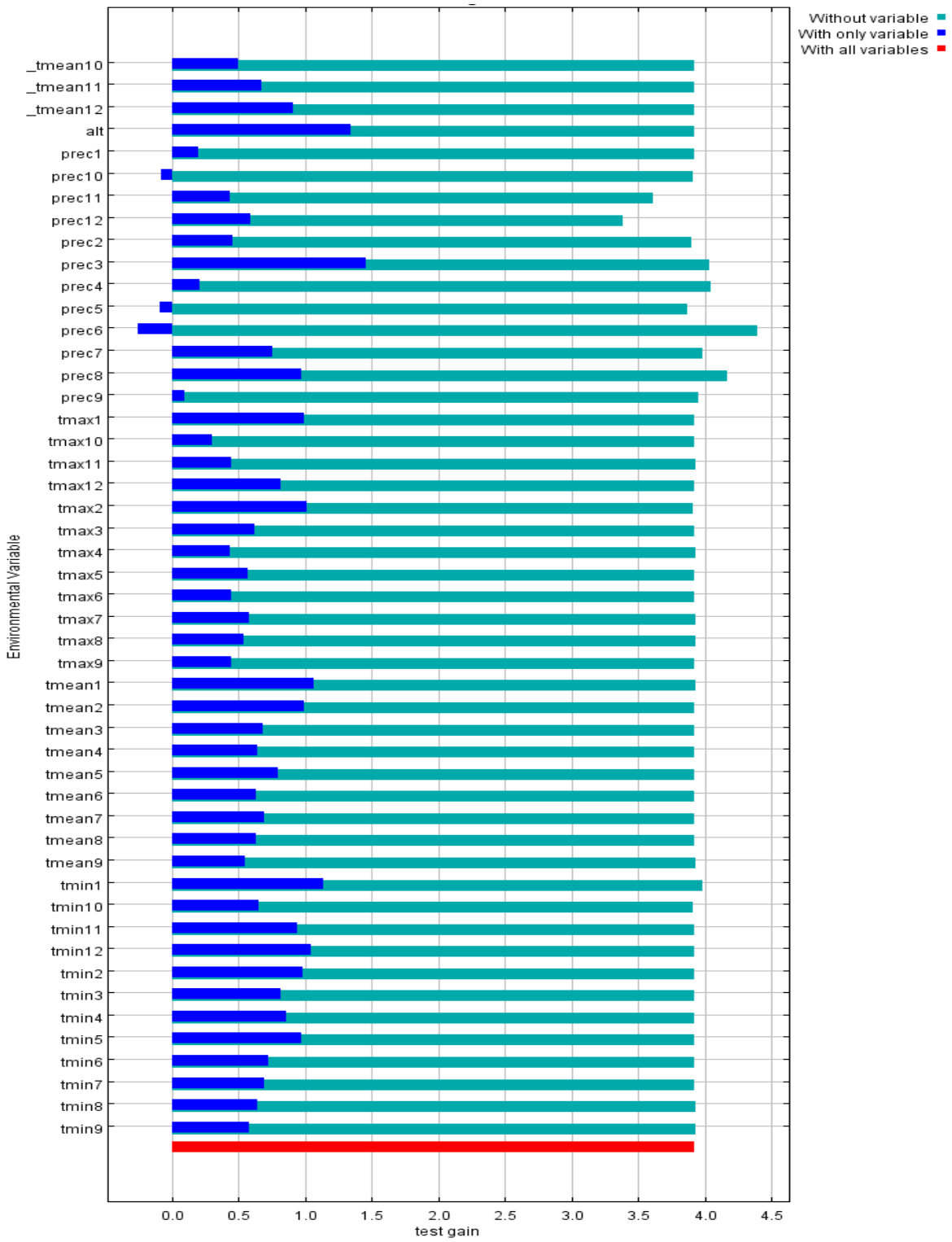


Figure - 2.7

Figure - 2.7: Model-2. Result for the Jackknife test using test gain.

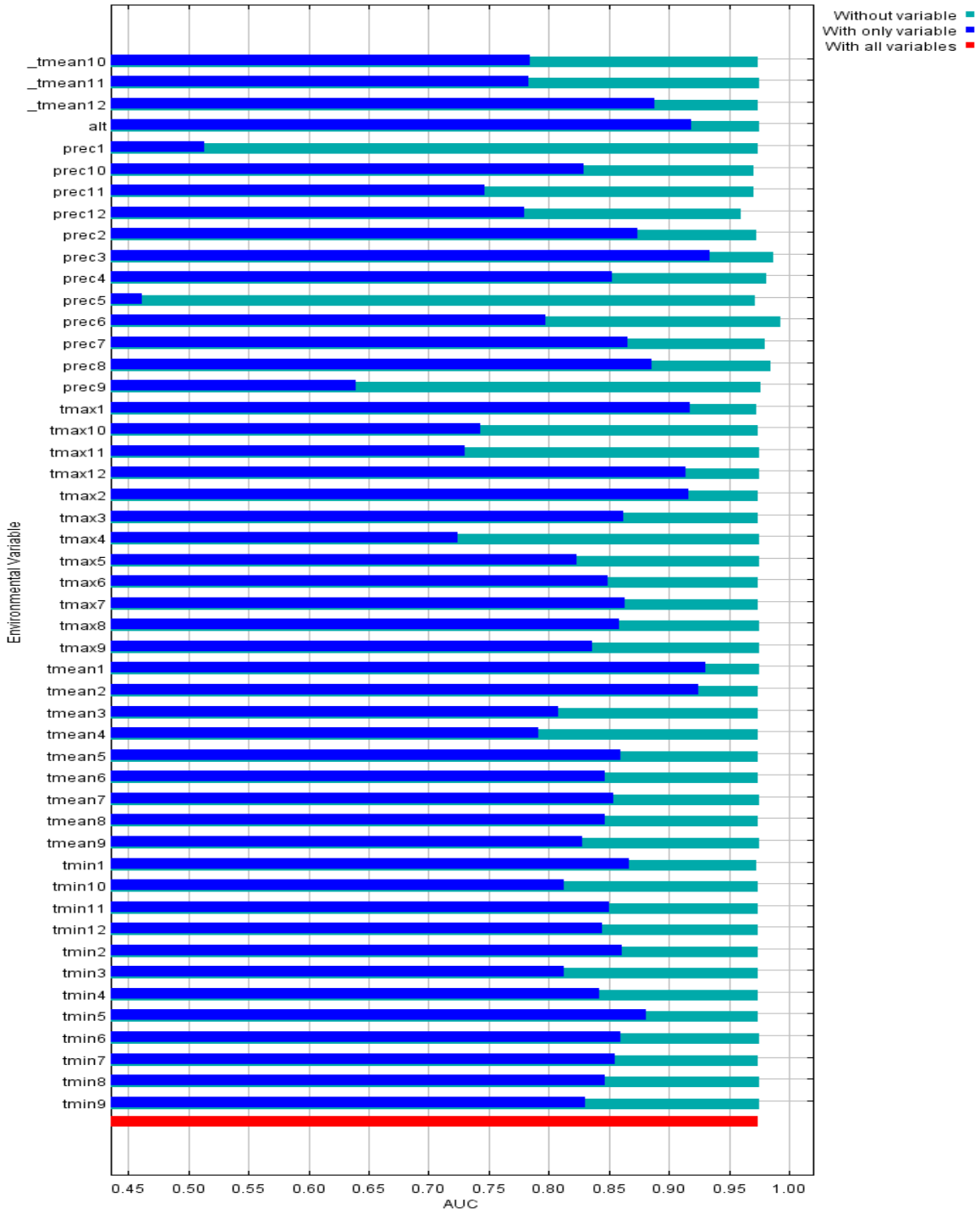


Figure – 2.8

Figure - 2.8: Model -2. Result for the Jackknife test using AUC on test data.

Jackknife test using AUC on test data (**Figure - 2.8**), the AUC plot shows that mean temperature in the month of January is the most effective single variable for predicting the distribution of the occurrence data that was left aside for testing, when the predictive performance is measured using AUC, though it was hardly used by the model built using all variables and the relative importance of BIO4 also increases in the test gain plot. The results from the second model also validates that temperature variables are playing an important role in the MaxEnt prediction model. In the present study it was found that out of the total 16579 Km^2 area of Nagaland ca. 1626 Km^2 falls under very high suitability threshold, 3369 km^2 under high category, 3468 km^2 under medium and low suitability and 8115 Km^2 falls under very low suitability threshold.

Model Validation and Authentication

Model quality was evaluated based on the prediction value of each of the occurrence points along with the climatic conditions of the predicted area. Jackknife method (Pearson *et al*., 2007) is used to develop the model, for validating model robustness, 12 replicated model runs was executed for the species with a threshold rule of 10 percentile training presence and employed cross validation technique where samples are divided into replicate folds and each fold used for test data. The model was able to predict suitable sites in the neighbouring Northeastern states of India and countries (**Figure - 2.9**). The high suitability threshold was validated in Manipur, Meghalaya and Arunachal with secondary occurrence data, the model prediction in neighbouring countries of Bhutan and Burma can also be supported by occurrence reports available from secondary sources. The model was able to predict suitable sites in neighbouring states and countries.

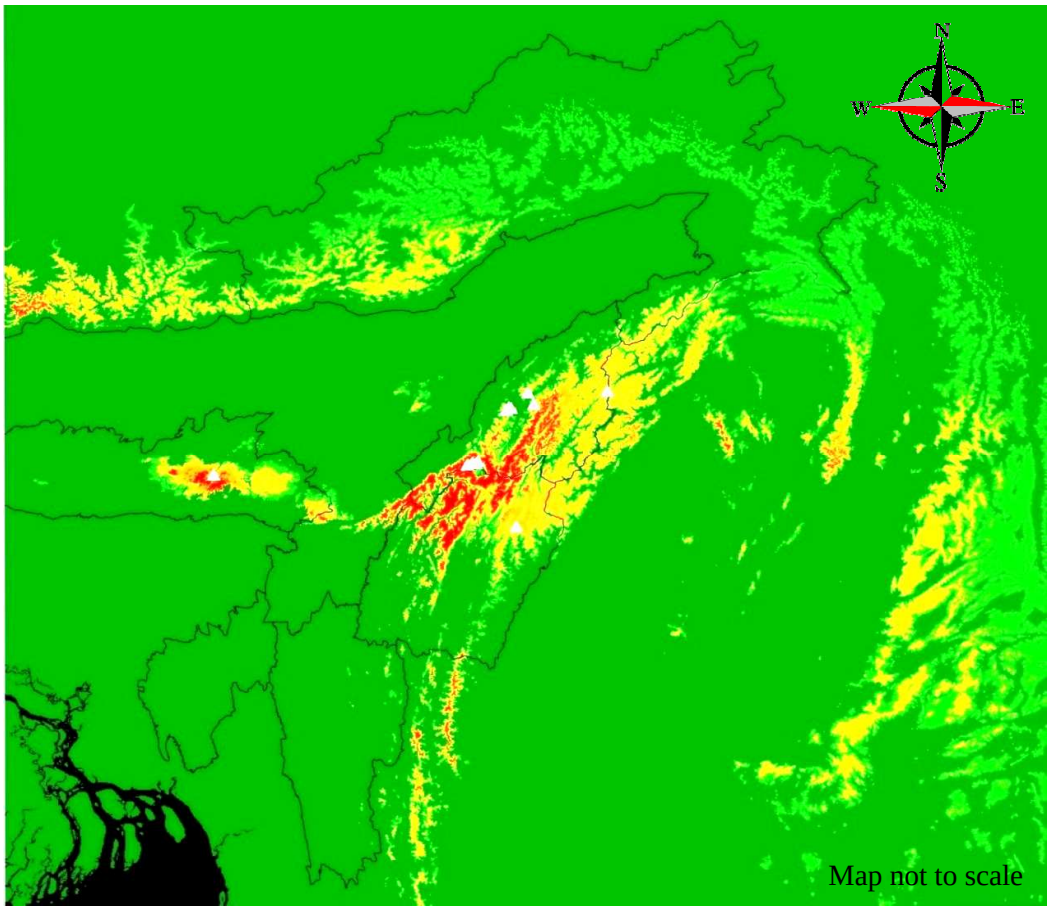


Figure – 2.9

Figure - 2.9: Distribution prediction map showing suitable sites predicted in nighbouring states and countries.

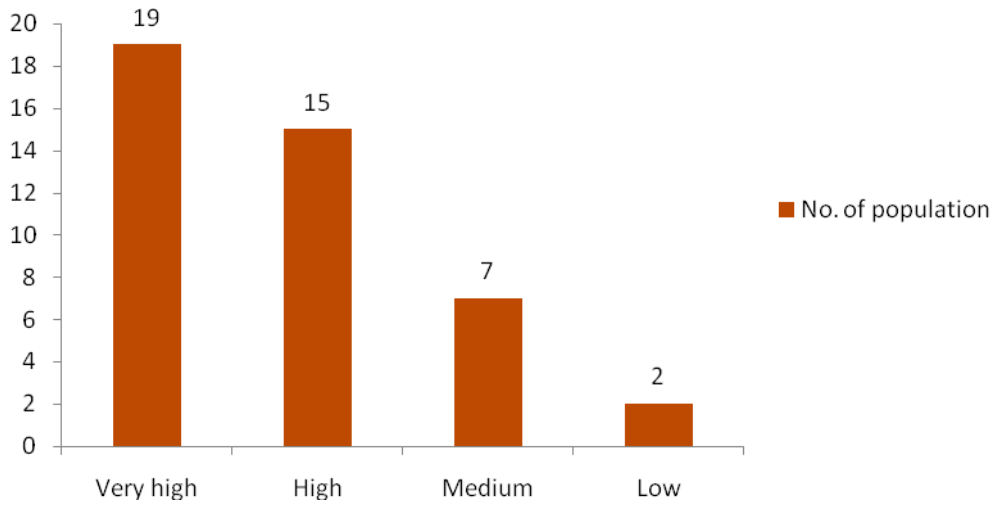


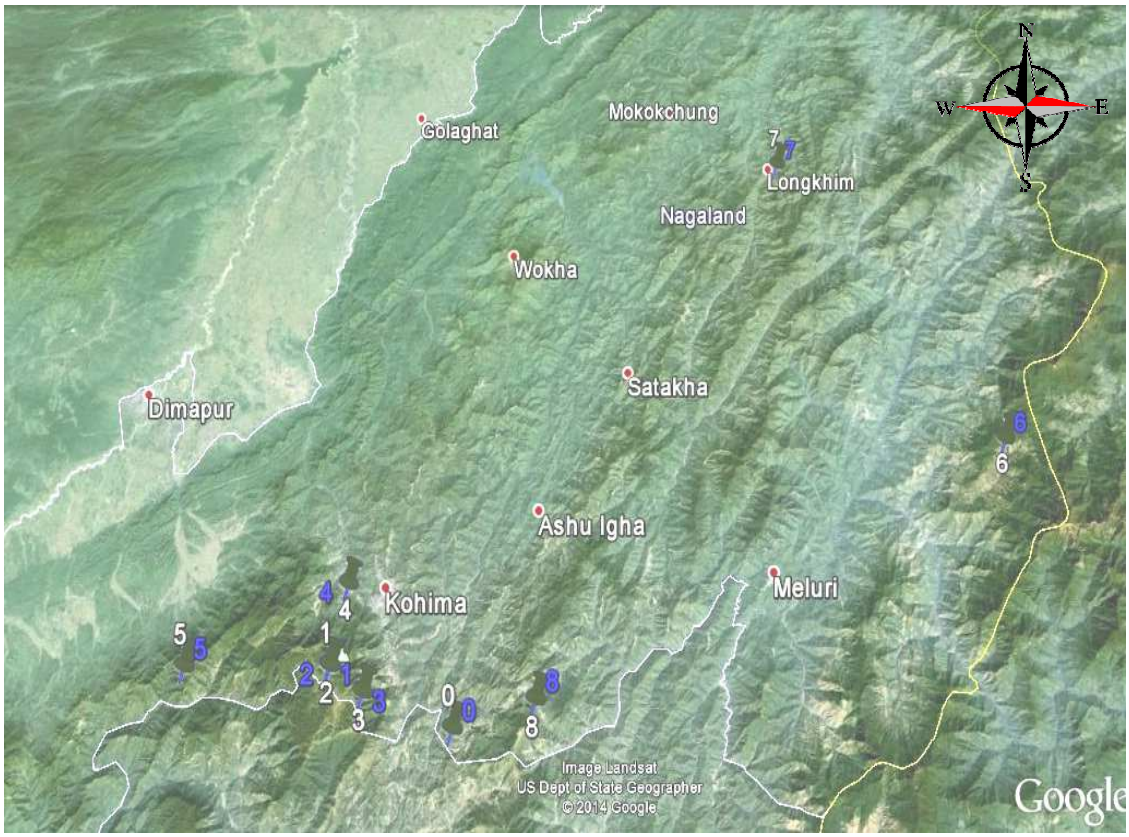
Figure – 2.10

Figure - 2.10: Number of population inventoried in different suitability threshold.

Table – 2.8: List of plants found in most of the area of occurrence

District	Area	Trees	Shrubs	Herbs
Kohima	Dzukou	*Rhododendron macabeanu, Betula* sp. *Prunus cerosoides, Lithocarpus* sp.	*Berberis wallichiana, Sinarundinaria griffithiana,Sinarundinaria rolloana, Gaultheria hookeri,Rosa sericea.*	*Aconitum nagarum* Stapf., *Dicentra scandens* (D.Don.) Walp, *Frageria* sp., *Potentilla lineate, Anaphalis contorta* (D.Don).
Kohima	Khezakhenoma	*Schima Wallichii, Quercus* sp. *Juglans regia, Lithocarpus* sp.	*Gaultheria hookeri,Sinarundinaria* sp. *Clerodendron* sp.	*Panax pseudogensing, Hedychium* sp. *Pteris* sp. *Piper* sp. *Boemeria* sp. *Frageria* sp *Girardiana diversifolia*
Kohima	Khonoma	*Schima Wallichii, Quercus* sp. *Juglans regia, Rader machera*	*Smilax* sp. *Urtica* sp.	*Dicentra scandens Pteris* sp. *Piper* sp. *Boehmeria* sp. *Girardiana diversifolia*
Kohima	Dzulekhe	*Schima Wallichii, Quercus* sp. *Juglans regia, Betula* sp.	*Urtica* sp. *Smilax* sp.	*Panax pseudogensing. Rubus* sp. *Calanthe* sp. *Pilea* sp. *Curculigo* sp.*Hedychium* sp. *Girardiana diversifolia,*
Wokha	Koio	*Schima wallichii, Quercus* sp. *Juglans regia*	*Smilax* sp.	*Mikinia* sp. *Curculigo* sp. *Hedera* sp. *Piper* sp. *Bidens* Sp. *Girardiana diversifolia,*
Wokha	Lungkhmuchung	*Schima Wallichii, Quercus* sp. *Juglans regia*	*Smilax* sp. *Urtica* sp.	*Piper* sp. *Pteris* sp. *Curculigo* sp. Hedychium Sp. *Pilia* sp. *Girardiana diversifolia,*
Wokha	Mt. Tiyi	*Schima Wallichii, Quercus* sp. *Juglans regia, Rhododendron* sp.	*Smilax* sp.. *Urtica* sp. *Clerodendron* sp.	*Hedera* sp. *Curculigo* sp. *Mikinia* sp. *Girardiana diversifolia*
Mokokchung	Lungkhum	*Schima Wallichii, Quercus* sp. *Juglans regia, Prunus* sp.	*Girardiana diversifolia, Urtica* sp. *Smilax* sp.	*Pomeria* sp. *Hedychium* sp. *Curculigo* sp
Tuensang	Pangsha	*Schima Wallichii, Quercus* sp. *Juglans regia, Mallotus macaranga, Rhododendron* sp.*Lithocarpus*	*Smilax* sp. *Urtica* sp. *Elastonia* sp.	*Pilea* sp. *Curculigo* sp. *Mikinia* sp. *Pteris* sp. *Girardiana diversifolia*
Zunheboto	Aizuto	*Schima Wallichii, Quercus* sp. *Juglans regia*	*Girardiana diversifolia, Smilax* sp. *Clerodendron* sp.	*Bidens* sp. *Curculigo* sp. *Rubus* Sp. *Hedychium* sp. *Eubatorium* Sp.

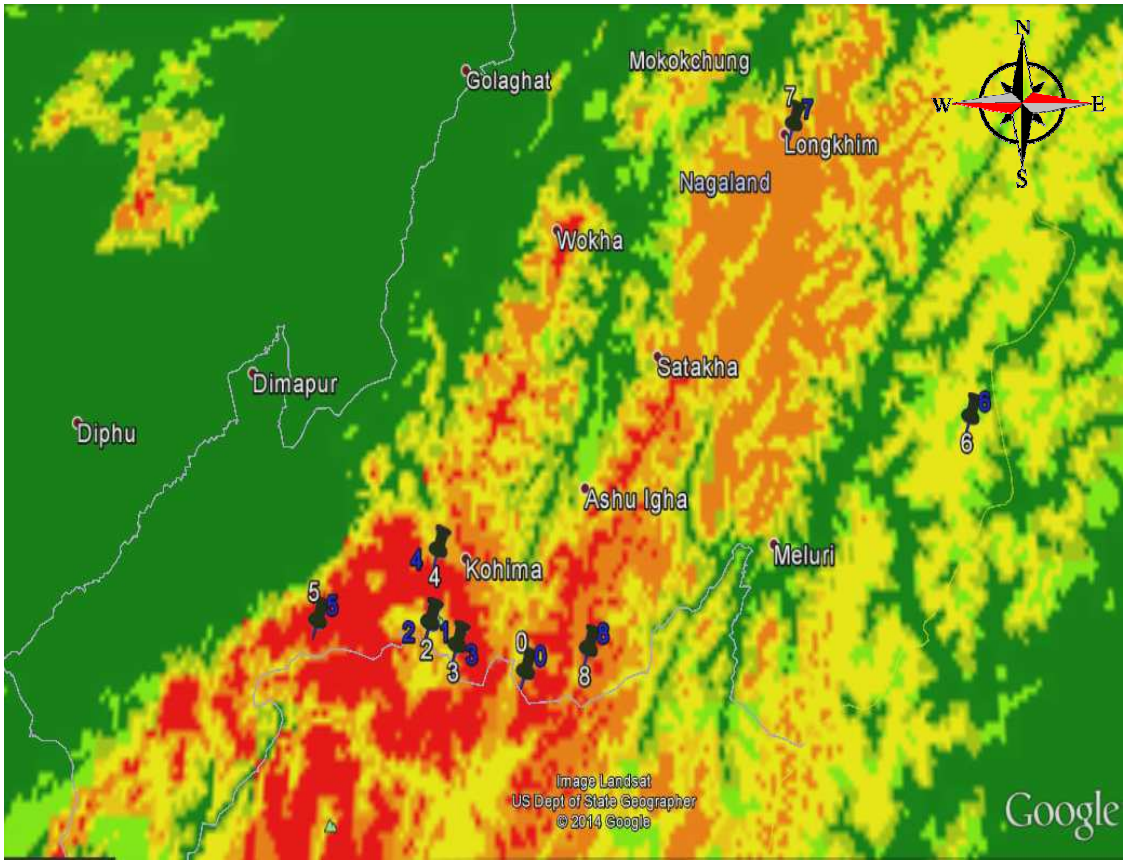
Primary ground truthing work was undertaken based on the prediction model to ascertain model robustness and nine sites with high prediction threshold were surveyed, the detected occurrence points gives a prediction value of and 0.7/1 (**Table - 2.5**) from stack created from the grid file of the model developed, thus establishing 70% success prediction. The model developed using only temperature and precipitation data was also able to correctly predict occurrence in Dzukou valley, an area not predicted by the model developed using the 19 bioclimatic variables. Both the models developed bring out important information bioclimatic factors determining the survival and regeneration of the species. A total number of 294 plant individual from 18 plant populations having 43 sub populations were inventoried and 19 of these sub populations were found in very high suitability prediction threshold, 15 in high, 7 sub populations in medium and 2 of the sub population in low prediction threshold (**Figure - 2.10**). The occurrence of the species is found in patches this could be due to absence of dispersal agents and no biological agents for this purpose has been reported, as such the seeds are dispersed by gravity and are spread in the vicinity of the mother plant and thus the occurrence of the population in patches is observed. The area of occurrence was mostly undisturbed forest and well shaded by canopy cover of trees belonging to *Schima wallichii*; *Quercus* species; *Juglans regia* and *Lithocarpus* sprcies and two populations was found under bamboo forest. Some of the main associate species (Table - 2.8) found in almost all the areas except Dzukou are *Urtica* species; *Girardiana diversifolia*, *Smilax* species; *Curculigo* species etc.



Map not to scale

Figure – 2.11

Figure - 2.11: Google earth map showing the areas validated (*Numbered in Blue)*.



Map not to scale

Figure – 2.12

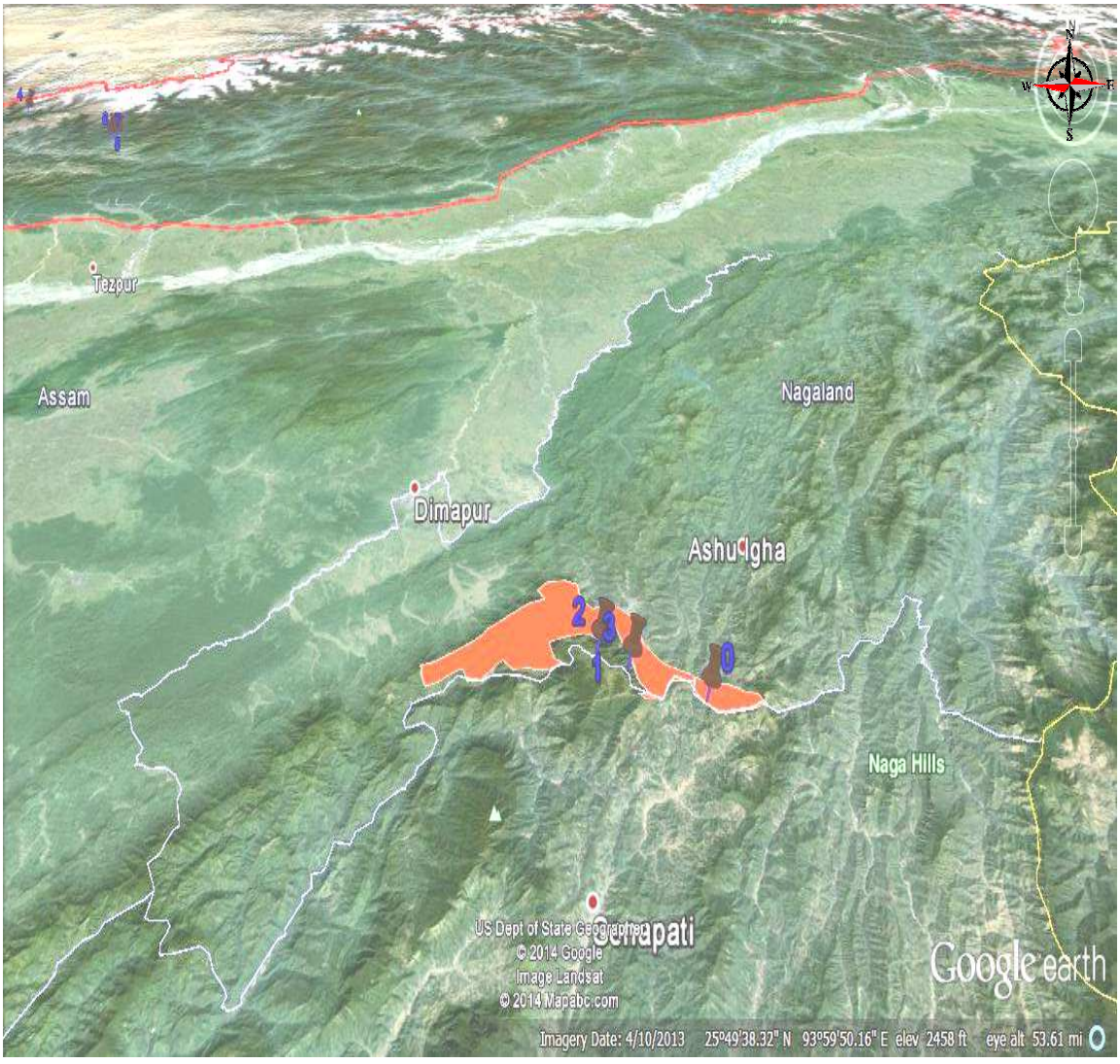
Figure - 2.12: Map showing the different levels of prediction and the validated areas (0-1).

Conservation Planning and Prioritizing Areas for Re-introduction

The different populations have been observed in tropical, sub-tropical forest and temperate forest under canopy cover, either under well stratified tree covers or bamboo forest. Biological impacts like logging, Jhum and collection are some main factors that are bringing noticeable changes to the forest over a short period. Habitat range of the plant varies from a well shaded full canopy cover to partial cover with presence of a thick forest litter. High level of anthropogenic activities effects population dynamics and disturbs the fecundity of adult population. Effective studies on population dynamics and fecundity in nature could not be given an insight approach as the levels of biotic interference are too high and does not allow continues and regular studies of the population in question and viability of the meta-population is rendered almost negligible and efforts are on to design an alternate approach.

The spatially separated population occurs in forest types ranging from tropical, sub-tropical to temperate forest. This spatially separated population shares similarity in adaphic conditions like forest litter content, similarity in seasonal climatic variables like temperature and precipitation. These occurrence points all fall within a minimum winter temperature range 0-7^{o}C and a mild summer temperature ranging from 20-28^{o}C except for Dzukou which experiences below freezing point during winter months. The lower ranges of Japfu mountain forest belt having a high occurrence falls within very high prediction threshold offers sites for species conservation, ground truthing also revealed the existence of southern Angami village community forest, western Angami village community forest and some Chakesang village community forest (**Figure - 2.11; 2.12**). Superimposing the prediction map to Google earth has shown special reserve areas falling within high prediction threshold *viz*. Pulie badze wildlife sanctuary, Khonoma nature conservation and Tragopan sanctuary, Dzukou valley, southern and western Angami

village community reserve forest whose presence were validated through ground truthing (**Figure - 2.13**) . These forests will serve as excellent sites for *in situ* conservation and possible re-introduction for species recovery.



Map not to scale

Figure – 2.13

Figure - 2.13: Priority area projected for conservation and possible re-introduction (Khezakhenoma, Dzulekhe and Khonoma area highlighted).

Discussion

The MAXENT model clearly indicates the importance of temperature in the development of the model, the first model clearly defines the usefulness of temperature in the prediction model which is validated by the second model developed using only monthly temperature and precipitation. The need and importance of cold temperature and temperature stratification for the species can be also confirmed by Zhou *et al.* (2003) in their paper '*Low-temperature stratification strategies and growth regulators for rapid induction of Paris polyphylla* var. *yunnanensis seed germination'*. They studied the effect of low temperature on the germination of *Paris polyphylla* seeds and they found that a stratified exposure to low temperature of 4^{o}C and 22^{o}C for a period of about 6 months is required to break the dormancy of the seeds, The climatic parameters identified by the model as a key in determining the species survival was cross referenced with an experiment performed by Zhou *et al.* (2003). The experiment mimics natural conditions and the seeds of *Paris polyphylla* were exposed to two subsequent set of temperature treatments at 4^{o}C and 22^{o}C for 112 days. In nature *Paris polyphylla* seeds remain dormant up to 2 years with occurrence of post dehiscence seed maturation (Li, 1986). The seeds starts germination in about 160 days with a germination rate of 95.3%, treatment with PGR without cold temperature does not break the seed dormancy and a very interesting phenomenon was observed in the high predicted areas in both the models i.e. the model developed using the 19 bioclimatic variable and the model developed using only temperature and precipitation data. The two models result clearly identifies the variable which plays an important role for determining the survival and multiplication of the plant species (**Table - 2.6 and 2.7**). The two models were able to bring out significant result in identifying the natural temperature stratification controlling seed germination in nature that is comparable with Zhou *et al.* (2003). The significance of this minimum temperature in the month of December and January can be compared with the post dehiscence *Paris*

polyphylla seeds. The two models stressed out the importance of cold climatic conditions and different seasonal temperature post dehiscence. In nature the areas which are able to provide these climatic requirements will be ideal locations for their conservation as well as re-introduction, significantly the prediction model developed was able to predict the areas having a climatic conditions similar to the above experiment performed by Zhou *et al. (*2003). This study clearly indicates the need of a stratified low temperature treatments for the seeds to germinate. Ground truthing revealed that the prediction model clearly shows a pattern similar to this experiment in the high prediction threshold areas. Figure - 2.14 shows the number of training data which falls in the climatic conditions which the model prediction was based on. Seeds are dehisced in the month between September - October, and the areas which are found suitable has natural temperature stratification almost similar to the experiment indicating the need of low temperature stratification for seeds to germinate which gives the continuity of the plant species. Study on the highly suitable areas shows that low temperature treatment in nature occurs in the month of December-January with varying minimum temperature between 0^{o}C-5^{o}C and maximum temperature of 14-20^{o}C, and in the months between May-September experiences the warmest quarter (average temperature between 20-26^{o}C) with high precipitation corresponding with BIO18 (precipitation of warmest quarter) when the seed experiences high temperature and precipitation during the first year and plants breaks ground during the second year. The second model also validates the low temperature requirement by bringing out the correct period of the year coinciding with real world phenomenon and the two MAXENT model projects an almost similar prediction model. This study clearly reveals the effect of temperature along with precipitation as an important factor in the biological clock of the plant species, as success of the seeds to germinate gives continuity to the plant species.

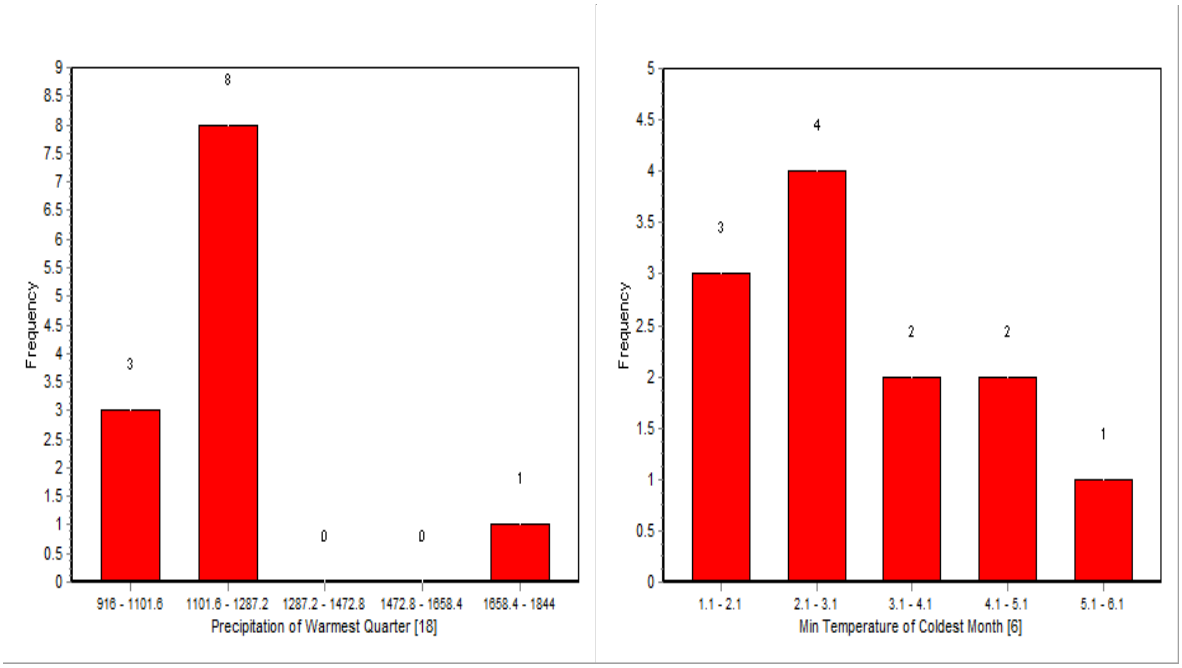


Figure – 2.14

Figure - 2.14: Histogram of training data with respect to the two most important climatic variables.

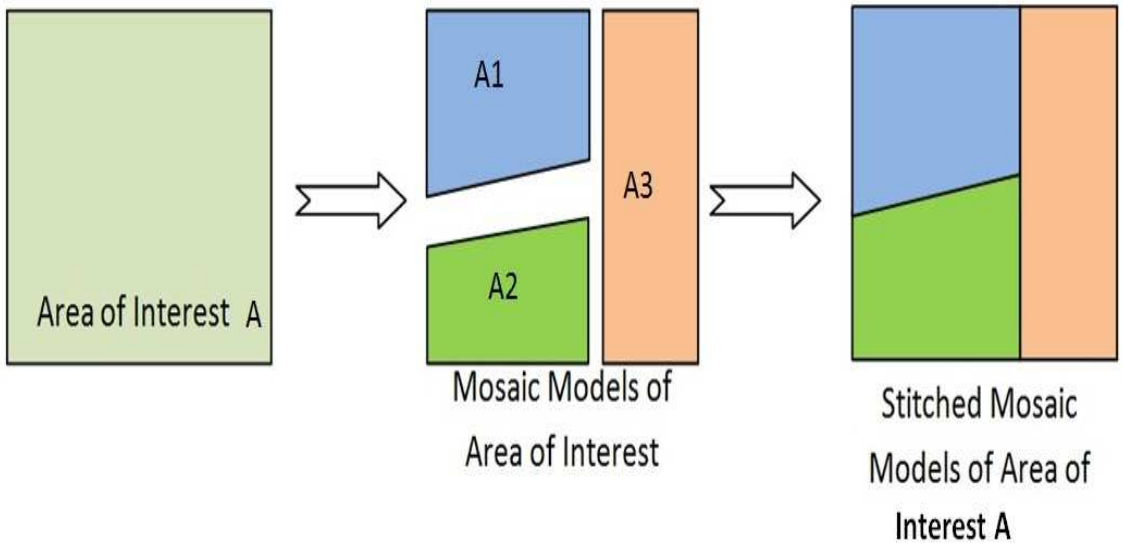


Figure – 2.15

Figure - 2.15: Fragmentation of sites into mosaic for prediction models.

The possibility of climatic threshold as a limiting factor in Niche modelling can increase for those species which are highly sensitive to change in climatic conditions. Prediction of suitable sites over a large area do not always gives usable models when the occurrence points are low (Pearson *et al.*, 2007). In this context the total area can be fragmented and models can be developed for each area and the mosaic of models can be stitched together (**Figure - 2.15**). This could serve well as a better model than encompassing a bigger area and this study shows the effectiveness of the model prepared using only 12 presence points over a small area, the present study also proves the effectiveness of prediction models developed using low occurrence points over a well defined area. As there is always the possibility of occurrence of the same species in a completely different set of climatic conditions, this could result in prediction of the threshold level between the two different sites having a completely different environmental condition as suitable as unsuitable area is included in the envelope and the model thus produced may result in over prediction thus will not be applicable. In such cases a concept of mosaic modelling where a particular area having different range of climatic threshold can be partitioned and models for each partition can be developed to reduce the influence of vast climatic differences within the area of interest on the model, example (Figure - 2.15) an area of interest (A) is divided into three parts (A1, A2, and A3) and prediction model for each of these areas is developed separately and the model thus obtained is stitched together as mosaic. In cases of donor and recipient approach to rehabilitation of species, the same species adapted in different climatic condition may not be suitable for re-introduction to a new area having different environmental set where earlier records of the species occurrence have been recorded. In this case, Dzukou valley having unique temperate climatic condition supports growth of the target species but the first model developed using the 19 bioclimatic variables fails to predict its occurrence

(Figure - 2.2) and plants collected from Dzukou valley were unable to survive when introduced in new area predicted suitable and in areas where the same species were present. This conditioning of plant species over a particular environment set could be a hindrance to re-introduction programmes and thus the concept of mosaic prediction models could help to overcome such problems, this concept however calls for more future in-depth studies.

This study also explores the potential application of Niche models in conservation. The target plant which is under high exploitation faces a threat of elimination from the natural habitat and this study provides a timely intervention for prioritization and conservation of the plant species in the high suitability areas. Introduction of plantlets in the selected sites also gives a good authentication of the ability of the MAXENT model in determining the suitability areas with respect to the selected climatic conditions. During the course of the study some major factors causing a rapid decline in the population of the plant species were identified. Major portion of this factors are biotic, mostly anthropogenic. This includes very high illegal collection rate in all the areas surveyed due to the potential market value of rhizome. Due to its slow growth cultivation of the species was not considered as an attractive alternative by the local peoples thus plants are collected in wild and very fewer efforts are done to cultivate the plant species. Apart from illegal collection and trade another major factor posing high threat is Jhuming and forest fire as it destroys vast expanse of forested areas and along with the destruction of forest, the habitat as well as the plant species is destroyed, unplanned developmental works like construction of Agri-link roads in villages lacks expertise, thereby causing more destruction to habitats. Anthropogenic activities leading to loss of adult plants lowers the fecundity and thus low fecundity cannot be related to natural conditions alone. The levels of exploitation in terms of excessive collection cannot be studied in detail due to the

unwillingness of dealers and local people to divulge information. Some natural factors like inability of flowers to produce seeds or failure of seeds to germinate is observed but these factors are overshadowed when compared with the magnitude of anthropogenic factors.

Summery and Conclusion

The work explores on the potential application of niche models to predict the occurrence of an economically important plant which have been highly exploited for its medicinal properties. During the present study MaxEnt was used to develop the climate suitability model as MaxEnt is a software specially designed to handle small sample size and presence only data. Two models were developed using two different sets of data and the model thus generated brings out interesting observation on the climatic parameters which are important determinants for the survival of the target plant. Climate parameters like minimum and maximum temperature in post dehiscence period plays a major role in the seed germination in nature and earlier studies mimicking this natural stratification has shown some comforting results. Model validation by ground truthing was also able bring out new population not only in the study site but also in neighbouring states and countries bordering the target study area. The present study clearly shows that climate suitability modelling can be employed for species specific recovery plan and in identifying sites for *in-situ* conservation and re-introduction. The plant population observed as a result of the ground truthing works based on the prediction model also strongly validates that climate suitability modelling can be successfully use for planning conservation works.

Chapter - 3

Niche Characterization and Distribution Prediction of *Vanda bicolor* in Nagaland through Modelling

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Recent Change in climatic condition has increased the pressure on plant species and many important species has been subjected to a lot of stress pushing them to the brink of extinction. In this aspect the issue of conservation has become a topic of utmost importance. Many plant species has been destroyed before they are documented or their value is realized, most of the medicinal and ornamental plants are highly exploited for their economic value. In this scenario visualizing a species' distribution in both geographical and environmental space helps to define some useful data that are crucial for species' distribution modeling knowledge of their distribution and the niche radius of target plant species will allow conservators to take up conservation works effectively for those species that are at risk. Species distribution modelling has become an important tool for conservation works as it provides an insight to the species geographical and climatic requisites and this data can be of immense help for conservationist.

To develop a suitable model for distribution prediction significant amount of data/variables are required. But for many species primary occurrence points are few and difficult to develop effective model. In the past few models were developed based on low sample size giving significant results viz., sample size of 4 (Loiselle *et al.,* 2003); sample size having a minimum of 2 (Ortega-Huerta and Peterson, 2004); sample size having a minimum of 4 (Pearson *et al.,* 2007); sample size of 16 (Adhikari *et al.,* 2012); sample size of 17 (Groff *et al.,* 2014). Useful models can be developed using low sample size with as few as 5-10 observations (Hernandez *et al.,* 2006).

Present study was based on few distribution points. In the present study identification of environmental data was done to bring out the climatic parameters within which the target species can persist and the geographical area where these set of environmental conditions are offered. Those areas offering similar climatic set as that of the training site are potential sites for their re-introduction taking into consideration the land-use pattern and biotic interaction in defining the species prevalence in the predicted regions as defined by Pearson and Dawson (2003). In cases where very few occurrence records are available due to low probability of detection (Pearson *et al.,* 2007), the occurrence records may not provide a sufficient sample size to establish the full range of environmental conditions occupied by the species to be identified but this is not necessarily a direct relationship between sampling in geographical space and in environmental space. It is quite possible that poor sampling in geographical space could still result in good sampling in environmental space. Past studies has shown that the predictive performance decreases significantly when samples size are low Pearson *et al.* (2007) and in the present study we investigated the performance of the models developed using low sample size. To enable the assessment of the prediction ability of the model developed

using small sample size we employed the Jackknife of Maximum Entropy (MAXENT, Phillips *et al.,* 2006) for presence only.

Materials and Methods

Algorithms Used

- **DIVA GIS 7.5**
- **ArcGIS 10.2.2**
- **MaxEnt 3.3.3e**

Different modelling algorithms have been applied to classify the probability of species' presence/absence as a function of a set of environmental variables. The present study employs MaxEnt algorithm to develop the model as MaxEnt software is based on the maximum-entropy approach for species habitat modelling. This software takes a set of layers or environmental variables (such as elevation, precipitation, etc.), as well as a set of geo-referenced occurrence locations as inputs, and produces a model of the range of the given species. The model for a species is determined from a set of environmental or climate layers (or 'coverage') for a set of grid cells in a landscape, together with a set of sample locations where the species has been observed. The model expresses the suitability of each grid cell as a function of the environmental variables at that grid cell. A high value of the function at a particular grid cell indicates that the grid cell is predicted to have suitable conditions for that species. The computed model is a probability distribution over all the grid cells. The distribution chosen is the one that has maximum entropy subject to some constraints: it must have the same expectation for each feature (derived from the environmental layers) as the average over sample locations (Phillips and Elith, 2011). All other spatial analysis works like data conversion, importing/exporting, mapping and visualization was done in DIVA GIS and ArcGIS.

Table - 3.1: Contributions of the environmental variables to the MAXENT models using the 19 Bioclimatic variables

Variable	Percent contribution	Permutation importance
BIO18	46.7	25.1
BIO13	16.2	1.4
BIO14	9.4	6.4
BIO19	8.5	10.5
BIO2	7.6	8.7
BIO7	4.8	9.5
BIO5	3.3	0.1
BIO4	1.2	18.5
BIO17	1.1	11.4
BIO16	0.4	0

Input Data

***Climate Data*:** Different types of environmental input variables have been used in species' distribution modelling. Most common ones are climate variables like (e.g. temperature, precipitation), topography (e.g., elevation, aspect), soil type and land cover type. These environmental variables may be either *continuous data* (data that can take any value within a certain range, such as temperature or precipitation) or *categorical data* (data that are split into different categories, such as land cover type or soil type). For the present study bioclimatic variables were obtained from Worldclim at 30´´ pixel resolution, which consist of an interpolated datasets of temperature and precipitation which are of primary importance for the plant to thrive and reproduce successfully at a particular area. Worldclim version.1 was developed by Hijmans *et al.* (2005). The climate elements considered were the 19 bioclimatic variables with different climatic parameters (**Table - 3.1**).

Occurrence Data: Four GPS points of the plant species, geo-referenced during primary ground surveys using GPS (eTrex 10) were used as occurrence points, all the occurrence points were subjected to quality test with respect to their positional accuracy was ascertained through Google earth, duplicates were identified and removed thus maintaining only one point within 1×1 Km^2 to avoid sampling bias which would otherwise favour the climatic of those sites where sampling is highly concentrated. The geo-referenced points are converted to Decimal Degrees (DD) format using Microsoft-Excel with a precision of four decimals from Degree Minute Seconds (DMS) format using the common formula; **Decimal degrees = [(Degrees (°) + Minutes (′) / 60 + Seconds (″) / 3600)] * H. Where *H = 1 when the coordinate is in the Eastern (E) or Northern (N) Hemisphere H = -1 when the coordinate is in the Western (W) or Southern (S) Hemisphere***.

As the number of presence points in below 20 (*i.e.* 12), 1.5x Inter Quartile Range (1.5 IQR) method of identifying outliers is applied to check for outliers based on climate data developed from the environmental data obtained from Worldclim Website at 30´´. All climate data are cross checked for resolution accuracy and corrected to 30" pixel resolution. For MaxEnt the coordinates are fed in longitude and latitude as MaxEnt can handle most coordinate systems provided that the Comma-Separated Value (.csv) file coordinates match the coordinate system of the spatial data layers.

Model Calibration: All modelling works was carried out using MaxEnt Version 3.3.3K as the present work was based on presence points only and has low sample size. MaxEnt can efficiently handle small sample size as described by Philips *et al.* (2006). All visualization was done in DIVA GIS 7.5.0, and all mapping works was carried out using ARC GIS 9.3. The model was developed using Jackknife method (Pearson *et al.*, 2007). This method is called *k*-fold partitioning. In this method the data are split into *k* parts of

roughly equal size ($k > 2$) and each part is used as a test set with the other k-1 sets used for model calibration. Thus, if we select $k = 4$ then four models will be calibrated and each model tested against the excluded test data. Validation statistics are then reported as the mean and range from the set of k tests (Fielding and Bell, 1997). An extreme form of k-fold partitioning, with k equal to the number of occurrence localities, is recommended for use with very low sample sizes e.g., < 20 (Pearson *et al*., 2007). This method is also called 'leave-one-out' since each occurrence locality is excluded from model calibration during one partition.

Model Validation and Authentication

For validating model robustness, 12 replicated model runs was executed with a threshold rule of 10 percentile training presence and employed cross validation technique for dividing the samples into replicate folds and using as test data all other parameters were kept at default following Adhikari *et al.* (2012). The AUC was graded according to Thuiller *et al.* (2005). The distribution potential of the model was classified into very low, low, medium, high and very high. To authenticate the model intensive field surveys was carried out in the different prediction threshold areas the presence and absence of the target species was noted with respect to the prediction map developed.

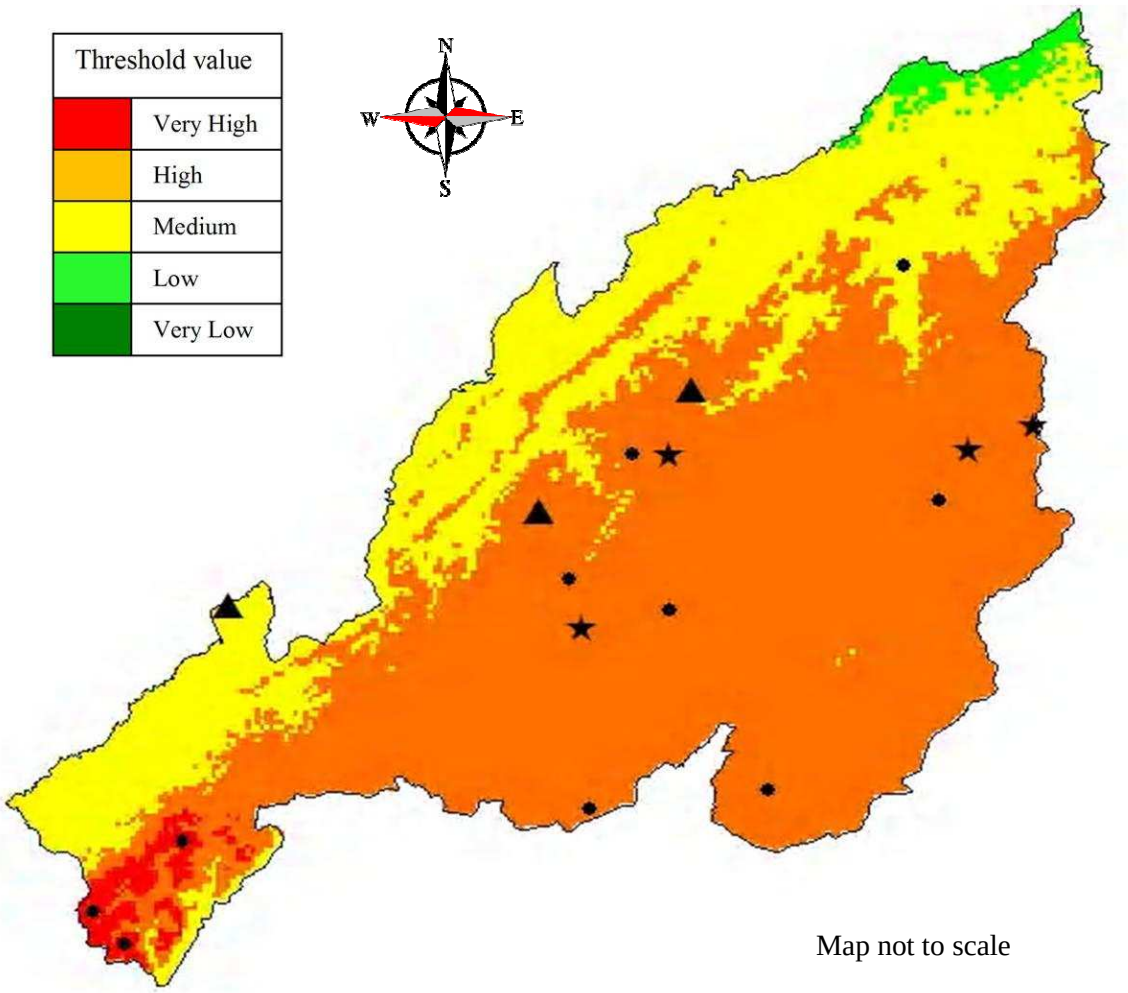


Figure – 3.1

Figure - 3.1: Distribution prediction map of *Vanda bicolor*. (★- Training sample, ●- Newly discovered occurrence, ▲- Introduced areas).

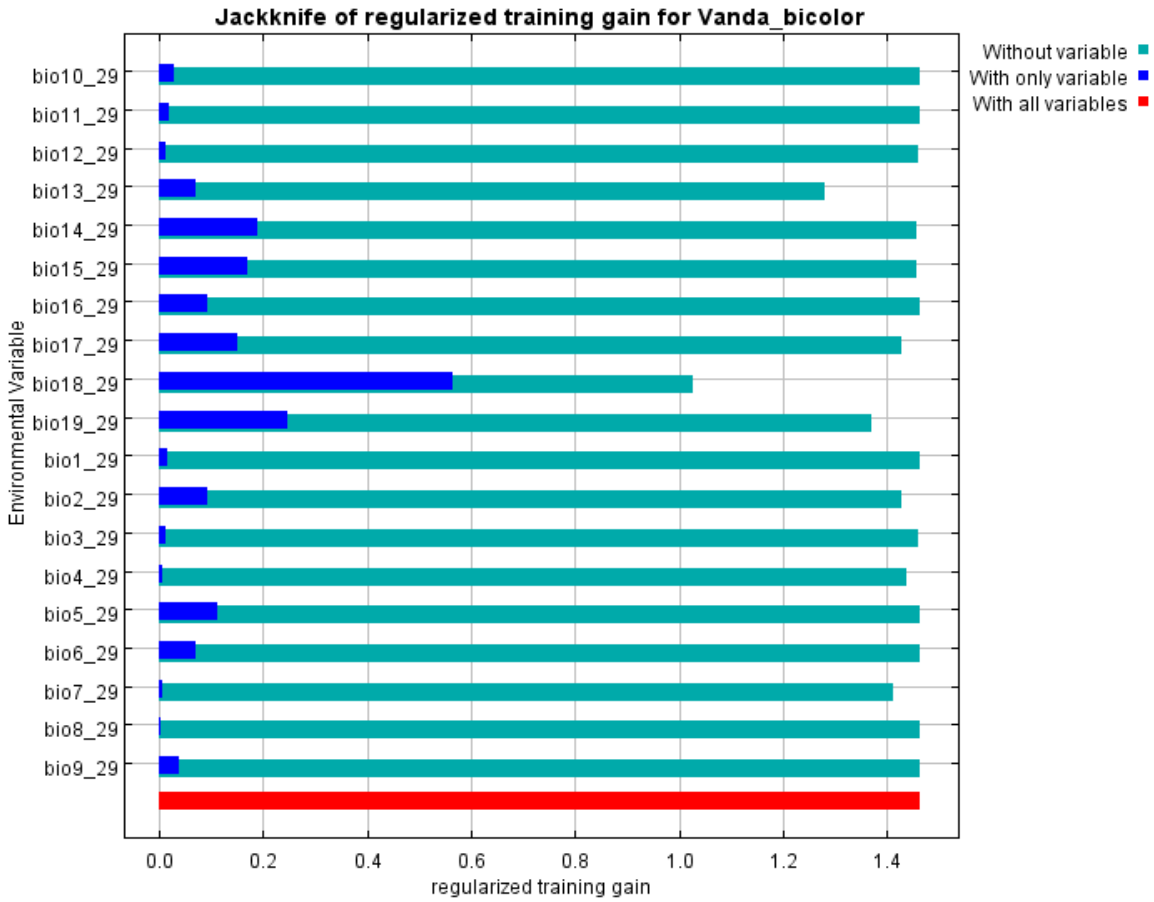


Figure – 3.2

Figure - 3.2: Result for the Jackknife test of variable importance.

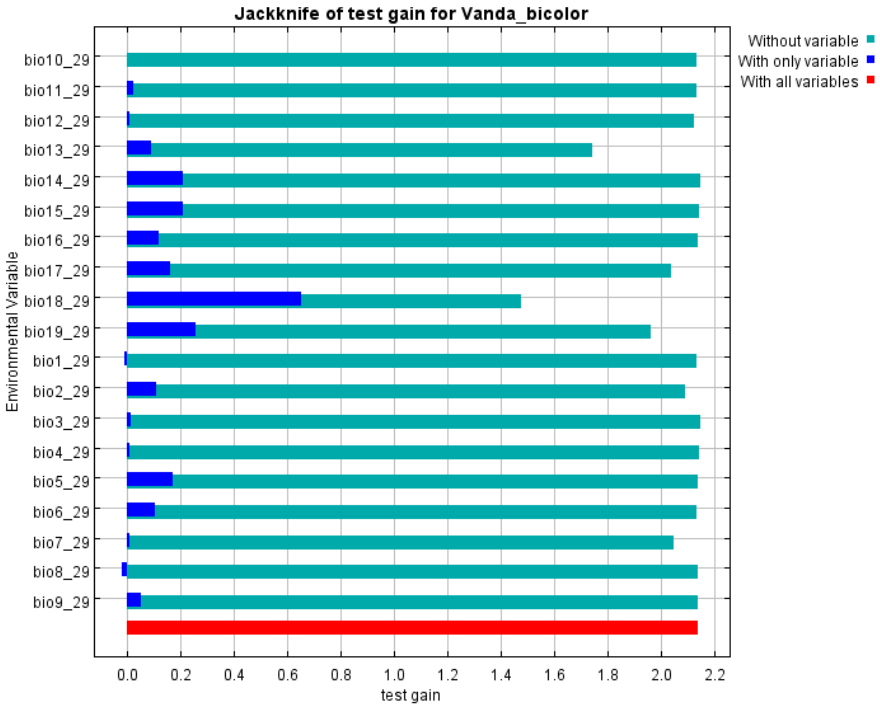


Figure – 3.3

Figure - 3.3: Result for the Jackknife test using test gain.

Results

The model calibration (**Figure - 3.1**) gives a test AUC of 0.984, with a standard deviation of 0.004. The AUC ranges from 0.5 for models that are no better than random to 1.0 for models with perfect predictive ability. The AUC test is derived from the Receiver Operating Characteristic (ROC) Curve. The ROC curve thus describes the relationship between the proportion of observed presences correctly predicted (sensitivity) and the proportion of observed absences incorrectly predicted (1–specificity). Thus, an AUC value of 0.7 means the probability is 0.7 that a record selected at random from the set of presences will have a predicted value greater than a record selected at random from the set of absences (Fielding and Bell, 1997; Pearce and Ferrier, 2000a; 2000b). In the present study estimates of relative contributions of the environmental variables to the MaxEnt model showed that BIO18 contributed the maximum (46.7%) followed by BIO13 and contributing 16.2and 9.4%.

MaxEnt jackknife test of variable (**Figure - 3.2**) importance shows BIO18 (Precipitation of Warmest Quarter) giving a reasonably good fit to the training data. The environmental variable with highest gain when used in isolation is BIO18, which therefore appears to have the most useful information by itself in the model. The environmental variable that decreases the gain the most when it is omitted was also observed in BIO18, which therefore appears to have the most information that isn't present in the other variables.

Same Jackknife test, using test gain instead of training gain (**Figure - 3.3**) also shows that Precipitation of Warmest Quarter as an important climatic variable in the test gain, the test gain plot also shows that a model made only using BIO8 (Mean temperature of wettest quarter) results in a negative test gain. The model thus is below a null model (i.e., a uniform distribution) for predicting the distribution of occurrences set aside for testing and the variables are not the useful as predictor.

Jackknife test using AUC on test data (**Figure - 3.4**), the AUC plot shows that BIO18 is the most effective single variable for predicting the distribution of the occurrence data that was left aside for testing, when the predictive performance is measured using AUC, though it was hardly used by the model built using all variables and the relative importance of BIO4 also increases in the test gain plot. These results establishes the importance of precipitation in the MaxEnt prediction model and the role of model development for MaxEnt to obtain a good fit to the training data with the Precipitation of Warmest Quarter defining better results on the set-aside test data (most useful variable as predictor) followed by BIO5 (Max Temperature of Warmest Period), BIO16 (Precipitation of Wettest Quarter) and BIO13 (Precipitation of Wettest Period).

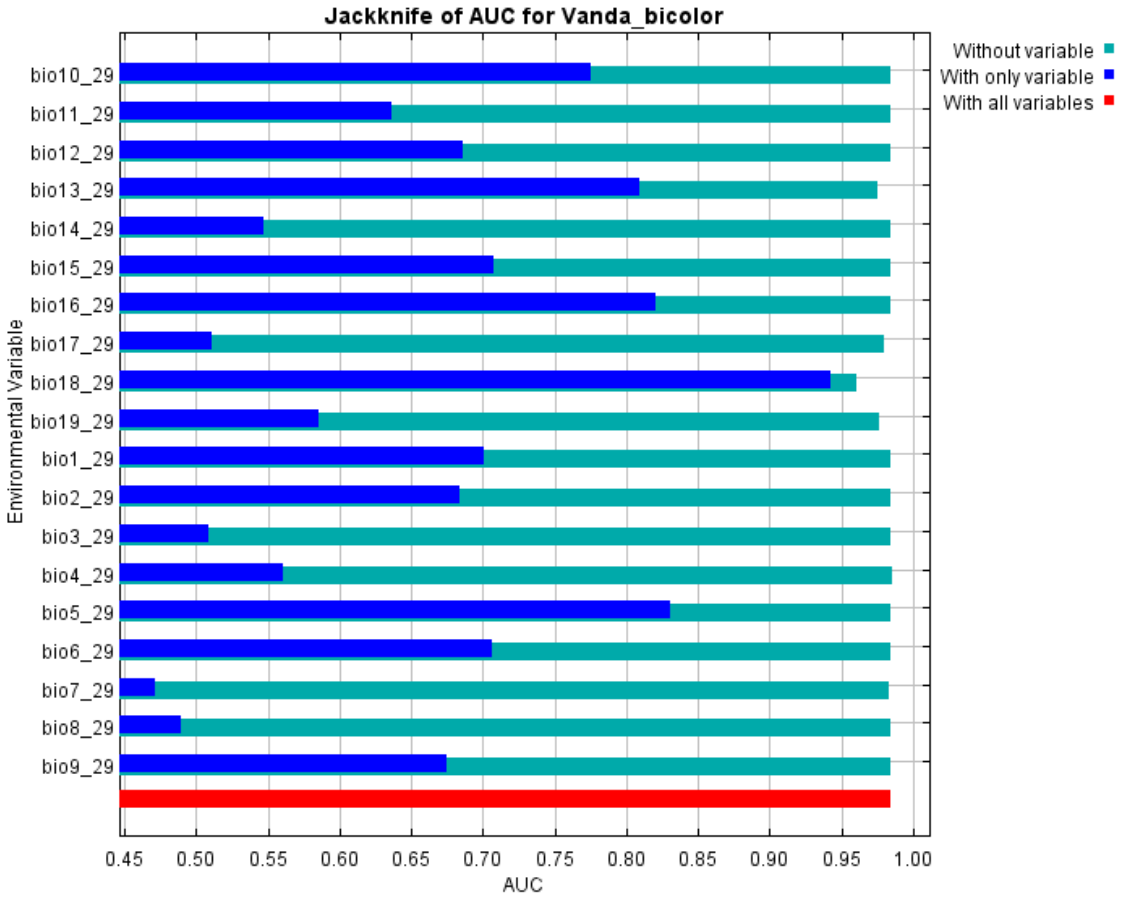


Figure – 3.4

Figure - 3.4: Result for the Jackknife test using AUC on test data.

Table - 3.2: Occurrence data in Nagaland acquired from ground truthing

Area	Longitude	Latitude	Prediction Threshold	Niche Status
Izheto	94.4200	26.2041	High	Disturbed forest, frequent Jhuming
Ghukimi	94.3110	25.9683	High	Disturbed forest, frequent Jhuming
Tsupfume	94.3443	25.5342	High	Disturbed forest, frequent Jhuming and forest fire
Aopao	94.8919	26.5591	Medium	Disturbed forest, frequent Jhuming
Ghokhuye	94.4832	25.9083	High	Disturbed forest, frequent Jhuming
Kengnyu	94.9535	26.1159	High	Disturbed forest, frequent Jhuming
Reguri	94.6557	25.5701	High	Disturbed forest, frequent Jhuming
Chisailhem	93.4831	25.3424	Very high	Disturbed forest, frequent Jhuming and forest fire
Nsong	93.5392	25.2798	Very high	Disturbed forest, frequent Jhuming and forest fire
Old Tesen	93.6391	25.473	Very high	Disturbed forest, frequent Jhuming and forest fire

Model Validation and Authentication

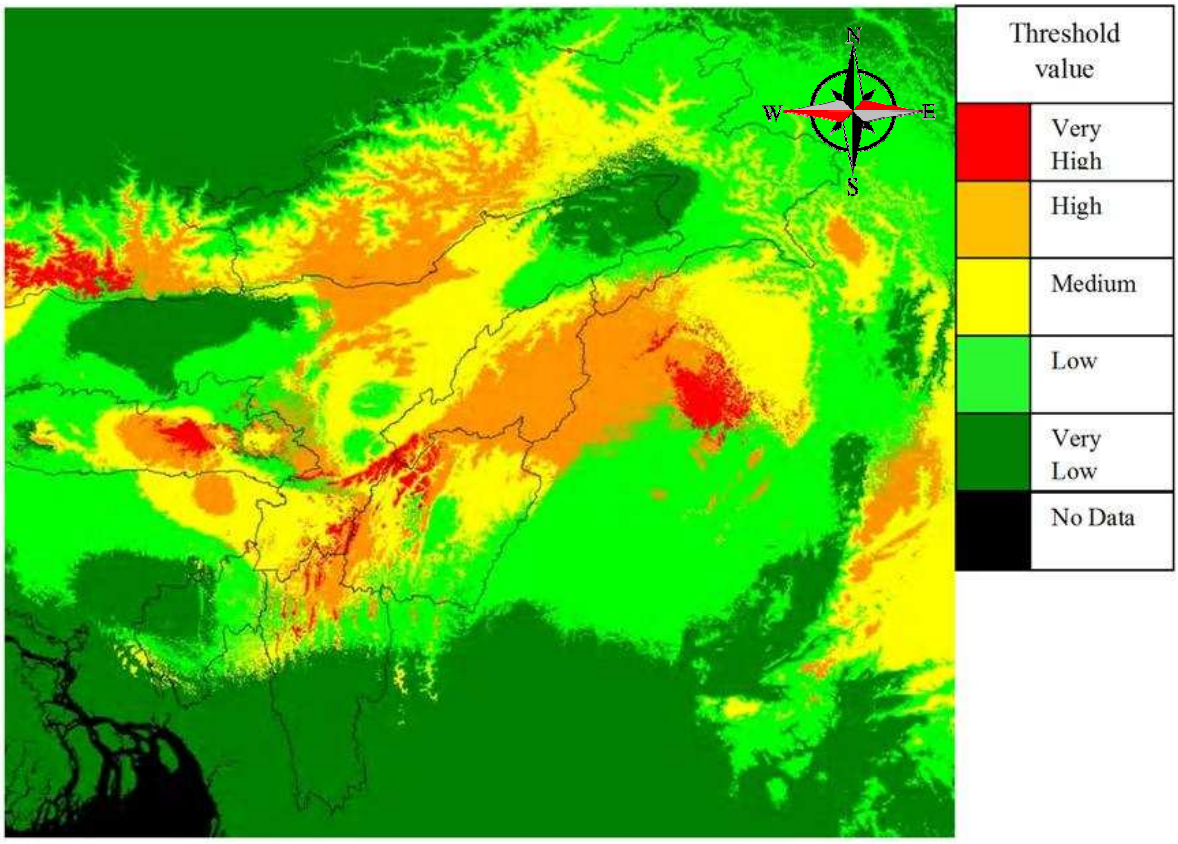
The model was developed using a very low occurrence points and most of the areas of Nagaland was predicted under high suitability threshold, thus to validate this, the model was subjected to intensive ground truthing and introduction in different prediction threshold to assess the model prediction ability. Target plant species was introduced in different areas and ground truthing works was carried out and it was realized that the distribution of the target plant species was highly threatened and very sparsely distributed in pockets. *K*-fold partitioning of test data and training data could not give usable model as the occurrence points are too low therefore Pearsons Jackknife method of leaving one out and assessing the predictive performance of each separate model was used. Jackknifing method was able to construct a workable model and ground truthing works by random selection of sites in different prediction threshold level give a significant result with 10 new occurrence records (**Table - 3.2**). The MAXENT model was able to give significant prediction results over a smaller area however, when the small sample size

data was used to predict over a larger area i.e., whole part of India and North Eastern region of India, the predication model becomes unstable and insignificant.

Though the model was developed using only four training sites, it was able to predict suitable sites in the neighbouring North-eastern states of India and countries (**Figure – 3.4**). The high suitability threshold was validated in Manipur and Arunachal, with secondary occurrence data, the model prediction in neighbouring countries of Bhutan and Burma can also be supported by occurrence reports available from secondary sources. The ability of the model to predict all the suitable sites over larger areas might be lowered as the training points are very less and confined over a smaller area (i.e., Nagaland), The model however shows a more robust prediction outside the target area in Bhutan and Myanmar (**Figure – 3.5)**.

Conservation Planning and Prioritizing Areas for Re-introduction

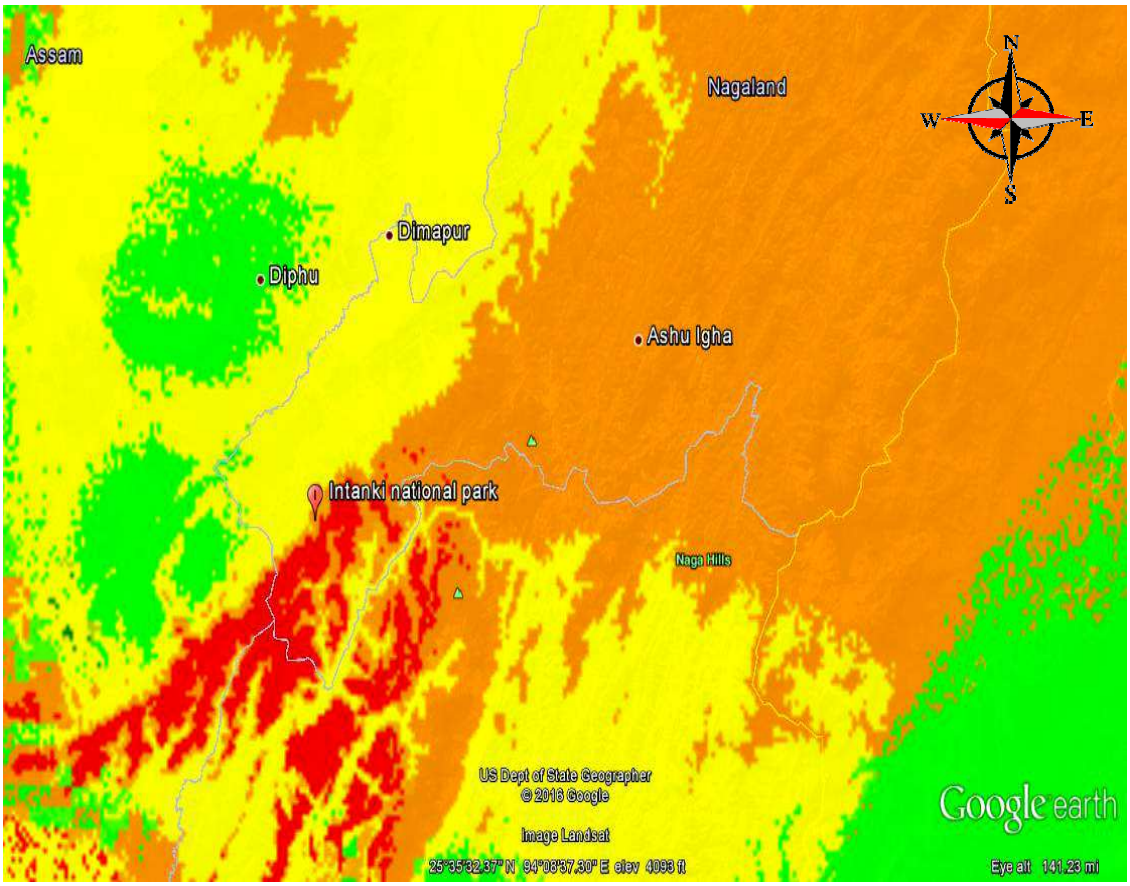
During the present study it was observed that most of the occurrence areas are under high biological disturbances like logging, Jhuming and forest fire and these are some of the factors that are bringing noticeable changes to the forest over a short period. This spatially separated population shares similarity in host plant and seasonal climatic variables like precipitation and temperature. Most of the areas are under high suitability threshold but are under high anthropogenic disturbances and only a small portion of the study in very high suitability threshold a falls under undisturbed area and interestingly Intanki National park fall under very high suitability threshold (**Figure - 3.6**). Introduction of species to random forests will proved to be futile if careful assessment of the forest condition is not done areas like Intanki national park will serve as excellent sites for in-situ conservation and possible re-introduction for species recovery.



Map not to scale

Figure – 3.5

Figure - 3.5: Distribution prediction map of *Vanda bicolor* showing prediction in the neighbouring states of the target area.



Map not to scale

Figure – 3.6

Figure - 3.6: Prediction map of *Vanda bicolor* showing very high suitability threshold which is part of Intanki National Park.

Discussion

The study was able to produce significant prediction models using very small sample size over a defined area, which has been validated statistically and though ground truthing. Earlier studies on development of models using low sample size has also reported effective models by using sample size of minimum 4 and 5 study on cryptic geckos (Pearsons *et al,* 2007). A testing of four modelling methods (Bioclim, Domain, GARP, and MaxEnt) across 18 species with different levels of ecological specialization using six different sample size treatments and three different evaluation measures revealed that MaxEnt was the most capable of the four modelling methods in producing useful results with sample sizes as small as 5, 10 and 25 occurrences. MaxEnt also predicted the largest area of all modelling methods at sample size 5 and remained fairly level at sample sizes of 25 and above (Hernandez *et al.,* 2006). In the present study MaxEnt was used to develop the model using very low sample size over a smaller area of 16,579 Km^2 to predict the climatic suitability of the target species. The model was able to bring out interesting insights on the climatic parameters which are playing an important role in the survival of the target species; the model defining precipitation as the most important predictor for the model. During field survey it was observed that occurrence areas are mostly hot and humid with high rainfall and the possibility of precipitation playing an important role in maintaining the population of the target species is quite relevant. The basic target for conservation works are on those species that are under high threat and those species in high threat category usually have low occurrence and it will be insignificant for conservationist unless working models are developed for these threatened species. In this study only climate data was used as our target species is plant species and climate plays a major role for its well being however, this does not negates the possibilities of anthropogenic and other biological factors contributing to the habitat

loss and low occurrence of species. The predication ability of the model with low sample size over a smaller area can be used to develop a mosaic of prediction models in areas where occurrence points are less and are in considerably distant pockets. Our study was able to give a success rate of 70% (calculated on stack developed using the MAXENT prediction map threshold value over the area of occurrence) with just 4 sample over a small well defined area. Introduction of the target plants to high and medium prediction threshold area (Wokha- 94°15′29.72″E, 26°05′47.10″E; Prediction Threshold -High; Mokokchung- 94° 31′22.25″E, 26°19′34.72″N; Prediction Threshold - High; Dimapur- 93°43′13.00″E, 25° 55′13.56″N; Prediction Threshold - Medium) gives normal flowering and seed formation. During ground truthing works it was found that most of the sites of occurrences fall in areas where there is frequent Jhuming and forest fire, the practice of Jhum cultivation and leaving it fellow for the next 5-10 years allows the regeneration of vegetation and the cycle is repeated and the survival rate of economically important plant species is much reduced. The present study thus could serve as a model for rehabilitations particularly for the conservation of economically important and rare plant species and gives ample scope to future researchers for more holistic studies on this approach and development of models with low training samples.

Summery and Conclusion

In the present study, the effectiveness of low sample size and climate data on MaxEnt model development and its usability in real world application has been validated statistically, though ground truthing and testing of sites by introducing plants to predicted sites. The model was able to bring out new insights on the climatic parameters which defines species survival, and successfully predicting new pollution in wild and those existing population in neighbouring states and countries with success rate of 70% (calculated on stack developed using the MAXENT prediction map threshold value over

the area of occurrence). Any conservation related works will be on those species that are under high threat and those species in high threat category usually have low occurrence and it will be insignificant for conservationist unless working models are developed for these threatened species and the present study gives a good example of how low sample size can be used to develop effective prediction models.

Chapter - 4

Propagation of *Paris polyphylla*

North East India is endowed with a unique plant biodiversity and is a part of the Indo-Burma hotspot and home to 8,000 flowering plants, which includes 40 species of gymnosperms, 500 species of pteridophytes, 825 species of orchids, 80 species of rhododendrons, 60 species of bamboo, and 25 species of canes (Chakraborty *et al.*, 2012). A study contacted by Botanical Survey of India (BSI) reported 200 plant species from Arunachal Pradesh being used for the treatment of 44 different ailments from, 286 plant species from Assam used for the treatment of 40 different ailments, 526 plant species from Nagaland known for the treatment of 83 disease and 194 plant species from Tripura for treatment of 50 disease (Mao *et al.*, 2009). The region though is a storehouse of many economically important plant species the level of anthropogenic activities is very high as a good fraction of the ethic people in this region are dependent on forest resource for economy, food, housing, fuel etc, and thus needs an urgent work on germplasm conservation of these economically important plants.

The present study was focused on the possibility of propagation of a highly medicinal plant *Paris polyphylla* using macropropagation techniques. *Paris polyphylla* is a perennial herb and loves to grow in shady and moist environment under thick canopy

cover. The plant species is highly exploited for its medicinal properties and is a highly prized forest resource among the indigenous tribal's for its economic value. This wonder herb is known for the treatment of different ailments, some of the ailments treated and the parts used include:

Rhizome: the rhizome is widely used as antihelmintic, antispasmodic, digestive, stomachic, expectorant and vermifuge (IUCN, 2004, Bhattarai and Ghimire, 2006). Powder from rhizome is used for fever and food poisoning. Rhizomes are also used for injuries from falls, fractures, convulsions and strains (Liang, 2000).

Root: Root paste is applied as an antidote for snake bites and poisonous insect bites and also to alleviate narcotic effects. Chewing a piece of the root is believed to heal internal wounds below the throat, and also it heals external wounds. It produces vasoconstriction in kidney, vasodilation in spleens and stimulates the isolated intestine (Dutta, 2007, Baral and Kumri, 2006). Pieces of roots are also fed to cattle with diarrhea and dysentery. Roots can also be used as analgesic, antiphlogistic, antipyretic, antitussive and depurative (Duke and Ayensu, 1985). A decoction of the root is used in the treatment of ulcers, diphtheria, epidemic Japanese B encephalitis, lymphadenopathy, tonsillitis, mastitis and rheumatism. It causes the subsidence of swelling, alleviates pain and relieves boils, carbuncles, sore throat and traumatic pain. It is used as a primary herb in the treatment of liver, stomach, nose, lung throat, and breast cancer in traditional Chinese medicine (Vassilopoulos, 2009).The whole plant can also be used as febrifuge.

The plant species is under highly threatened due to its excessive and unscientific collection and this issue unless addressed will lead to possible elimination in the region. *Paris polyphylla* propagation is difficult because of the high level of seed dormancy as the seeds requiring two winters and one summer in natural environments (Li, 1984), their regeneration in the wild is thus highly reduced leading to their decline in nature. Past

studies on their propagation has shed some light on the propagation of the species using tissue culture techniques, their regeneration through direct somatic embryogenesis from immature zygotic embryos (Roamai *et al.,* 2014a), and their regeneration using thin cell layer culture. The methods and techniques however require in-depth knowledge of biotechnology and the target of conserving the plant species by mass multiplication and re-introduction in wild may require huge resources and expertise. The present study thus explores to develop an easy to apply farmer's friendly procedures for the propagation of the species using macropropagation techniques. The study was thus focused on the regeneration of the plant species using rhizome cuttings and use of the rhizome as a mother rhizome.

Materials and Methods

Plant materials

Field surveys was carried out in Tuensang, Wokha, Kohima, Zunheboto and Phek districts and in every district plant materials were collected judiciously as dealing with highly threatened medicinal plant the availability of the plant material can be a limiting factor. Before collection proper permission was taken from the concerned village authority for sample collection. Plant material collection was done between September-November as during this period of the year bud emerges from rhizome and during collection the whole rhizome was not collected but a portion of the rhizome with bud is left behind to ensure its regeneration in its natural habitat.

Identification of sites

Experimental sites were chosen based on the prediction map developed as described in Chapter - 2 and this also serves as a test for the prediction map. Thus basing on the prediction map the target plant species was experimented in predicted very high suitability area *i.e.,* Site-1: Koio Village - $94^{o}18´0.333´´E$ $26^{o}07´0.108´´N$. To study the

impact of climate on the regenerative potential of *Paris poyphylla* two more sites with high suitability (Site- 2: Wokha village - 94°18´23.9´´E 26°06´59.5´´N) and predicted low suitability (Site-3: Lumami- 94°28´21.3´´E 26°13´32.0´´N) were selected and 100 rhizomes were introduced in each site.

Bed preparation

Moist and shady sites are chosen for bed preparation and selected sites are cleared of unwanted weeds and the soil is dug and the top soil (3-5cm) with humus is removed. The soil then is mixed with sand and NPK fertilizer locally available in the market. A drainage system is dug around the bed to avoid damping and water logging. The bed is provided with a shade as *Paris* loves to grow in shady areas under thick canopy cover.

Macropropagation

The plant material was checked for any infection before using it as planting materials and rhizomes with infections were discarded. The rhizomes were divided transversely according to number of growth rings/nodes, presence of buds, young lateral buds excised from mother rhizome and matured rhizomes with terminal buds. For each set 50 rhizomes were planted and 100 for matured rhizome. To study the affect of climate on the regeneration potential, 100 matured rhizomes (5-20 years) were introduced and the growth and response of the plants were observed. The responses of the plantlets in each site with respect to positive response and flowering are noted. Rhizomes are sowed at 10-15 cm apart at a depth of not more than 3 cm and after sowing the bed is covered with a mulch of the top soil mixed with humus and forest litter. Watering was done twice (morning and evening) in a day during dry seasons and once or no watering was done during rainy seasons.

Figure - 4.1

Figure - 4.1: Propagation of *Paris polyphylla* through rhizome splitting. A. Excised rhizome with lateral bud primordia (rhizome with nodular swelling), B. Excised rhizome with well defined lateral buds, C. Plantlet development from lateral branches with buds, D. Matured rhizomes, E. Development of lateral buds from matured rhizomes and F. Lateral buds detached from the parent rhizome.

Table - 4.1: Propagation of *Paris polyphylla* by rhizome splitting of different types of rhizomes

No. of rhizome planted	No. of growth rings/nodes on rhizome	No. of rhizomes responded	% of rhizome responded	Type of response
50	5 (without bud primordial)	0	0	Only formation of adventitious roots observed
50	6 (without bud primordial)	0	0	As above
50	7 (without bud primordial)	0	0	As above
50	Rhizome segment with bud primordia	13	26	Develops lateral buds
50	Rhizome segments with lateral buds	37	78	Develops into young plantlets
100	Intact matured rhizome	87	87	Grows into fully matured plants with sexual reproductive potential

Results

In the present study rhizomes of different categories were tested for plant regeneration/morphogenesis. It was observed that transversely segmented rhizomes with different numbers of rings/nodes without any pre-existing lateral buds remained recalcitrant except the formation of lateral buds and subsequently degenerated/rotten in the third year. Further there were three categories of rhizomes viz. 1. Rhizomes without any lateral bud primordia, 2. With lateral bud primordia (rhizome with nodular swelling) (**Figure - 4.1 A**) and 3. Rhizome with well defined lateral buds (**Figure - 4.1 B**). Further,

it was found that the rhizomes with lateral bud primordia mostly remained recalcitrant to morphogenesis and only 26% rhizome segments responded to morphogenesis. While, rhizomes with well differentiated lateral buds responded well and about 78% segments responded positively and formed healthy plantlets (**Table - 4.1; Figure - 4.1 C**). The lateral buds and branches were able to give rise to new plantlets but their development was restricted to formation of young plantlets and no signs of flowering were observed.

Further it was recorded that the matured rhizomes (**Figure - 4.1 D**) started forming new buds in the second year and pre-existing buds on the mother rhizome were able to develop into new lateral branches capable of giving rise to new individual plants which further could be excised and used as clonal planting material. During the study it was also observed that the intact matured rhizomes further gives rise to 1-6 new lateral buds. Besides these, number of nodes/ring in the excised rhizome segments exhibited pronounced morphogenetic effect. Rhizomes having more than 10 nodes or growth rings were observed to give better result in the development of lateral buds (**Figure - 4.1 E**). These lateral buds were detached from the parent rhizome (**Figure - 4.1F**) and planted during the subsequent years and normally grew into individual plantlets. Further it was also observed that if the lateral buds left un-detached from the parent rhizome, the offshoot rhizome mostly remains dormant and only few matured lateral buds give rise to new plantlet while still attached with the parent rhizome. These lateral buds can be excised and efficiently used as clonal planting material.

During the study the rhizomes introduced in site-1 (**Figure - 4.2, 4.3 A, B**) out of the 100 rhizomes 87% responded with 37% showing signs of flowering leading to subsequent seeds pods formation but only 21% were able to produce matured seeds and 13% of the rhizomes sowed fails to give any response. In site-2 (**Figure - 4.3 C, D**) out of the 100 rhizomes 76 responded and signs of flowering was observed in 34% of the

plantlets and 15% of the plantlets subsequently forming seeds pods but only 7% were able to produce matured seeds and 24% of the rhizomes sowed fails to grow. In site-3 (**Figure - 4.3 E, F**) out of the 100 rhizomes 47% responded and 12% showing signs of flowering with 4% carrying seeds pods but none of the seed pods reached maturity and most of the rhizome sowed i.e., 53% fails to give any response (**Table - 4.2**).

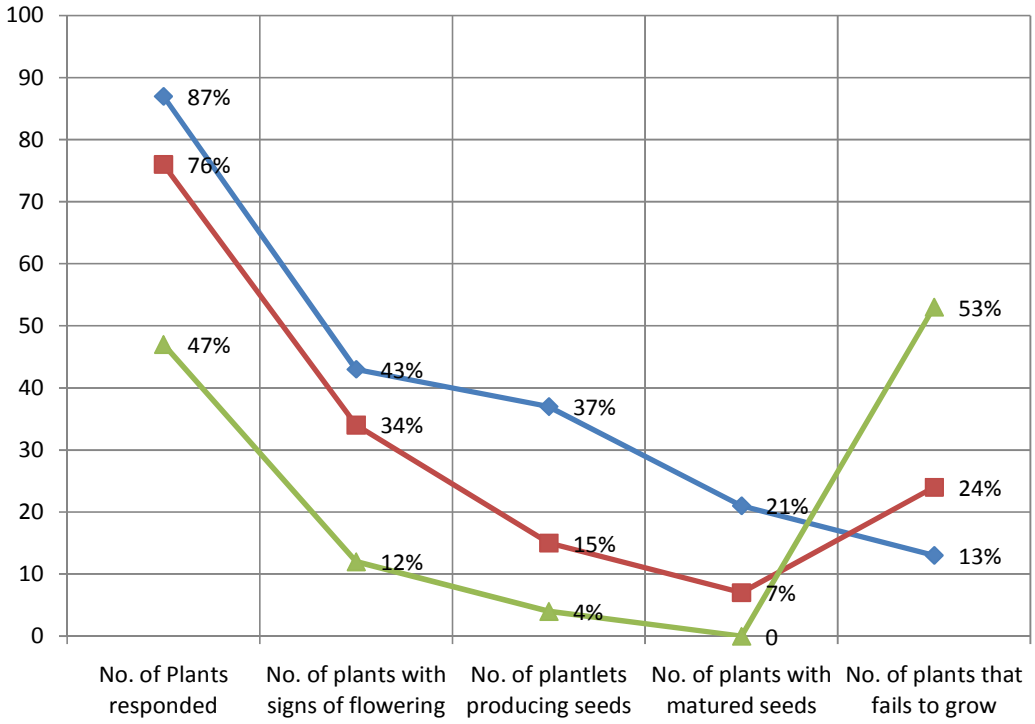


Figure - 4.2

Figure - 4.2: Graphical representation of the result of rhizome response to the different prediction threshold sites.

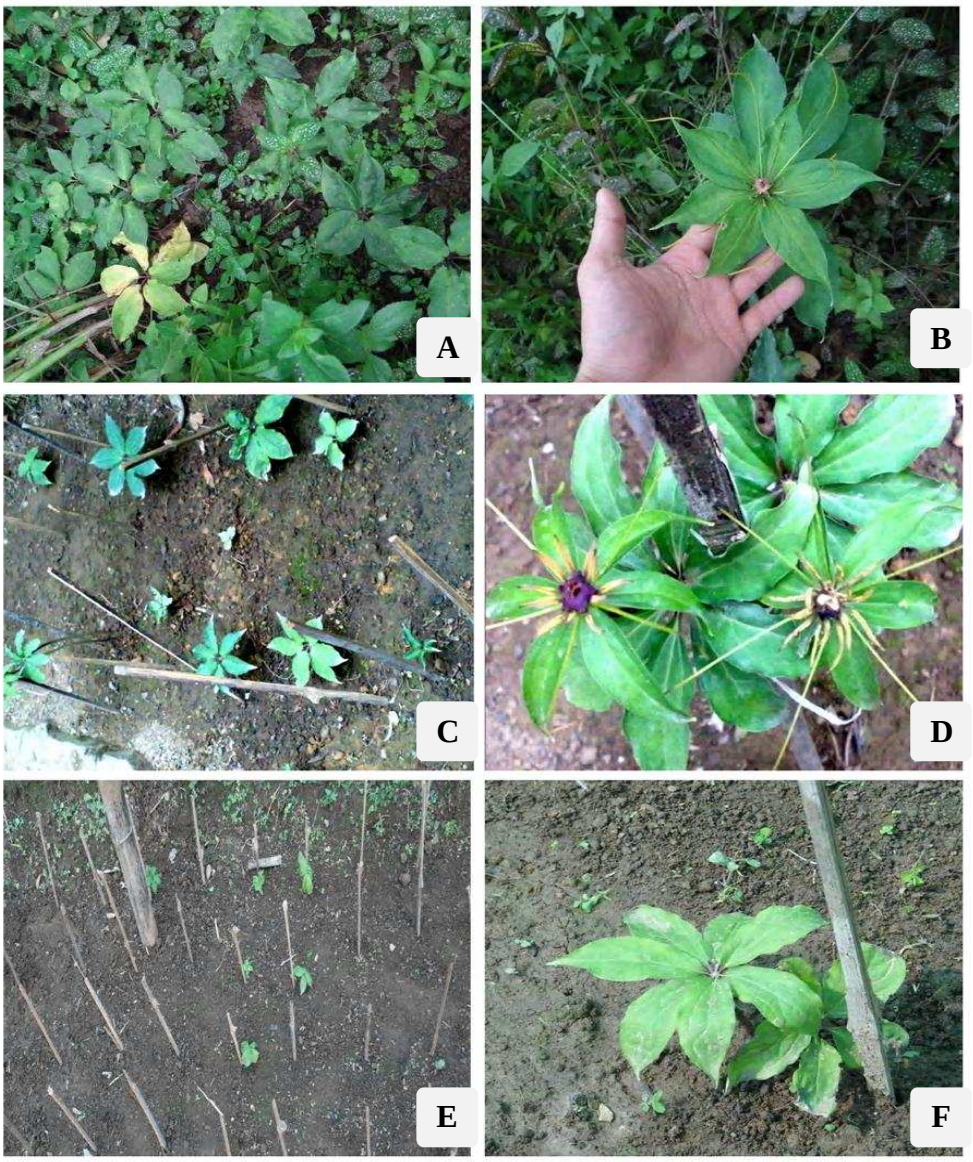


Figure – 4.3

Figure - 4.3: A- B. Plants introduced in site-1 (predicted very high suitable area), C-D. Plants introduced in site-2 (predicted high suitable area), E-F. Plants introduced in site-3 (predicted low suitable area).

Table - 4.2: Response of plantlets introduced to different prediction threshold sites with respect to positive response and flowering

Location	Suitability threshold	No. of plantlets Introduced	No. of plantlets responded	No. of plantlets flowering	No. of plantlets producing seeds	No. of plantlets with matured seed pods
Site-1	Very high suitability	100	87	37	21	13
Site-2	High suitability	100	76	34	15	7
Site-3	Not suitable	100	47	12	4	0

Table - 4.3: Plant population in the different sites under the study area

Area	Total no. of Population per Quadrate 1×1m^2 (mean)	No. of Adults Bearing Seedlings per Quadrate 1×1m^2 (mean)	No. of Saplings/ young plantlets per Quadrate 1×1m^2 (mean)
Khezakhenoma	20	5	12
Khonoma	12	3	7
Koio	15	2	9
Lungkhmuchung	13	1	8
Mt. Tiyi	10	1	7
Lungkhum	8	0	6
Pangsha	6	2	4
Aizuto	5	0	3

In site-1 success percentage of the introduced plantlet was high with plantlets showing flowering and formation of seeds the response of the plantlets was excellent despite the site was not pruned regularly and allowed to grow undisturbed with other native associates, however in site-3 which was predicted not suitable most of the plantlets did not show any signs of positive response and some of the few plantlets showing positive response also could not reach adult stage and only one plantlet was found flowering but seed formation did not occur (**Figure - 4.3 F**).

Discussion

The use of rhizome as a means of producing clonal planting material has been a common practice in vegetative propagation technique. Technically, rhizome cutting is a vegetative propagation technique using “rootstalk” also known as rhizome, and plants that spreads by rhizome are referred to as rhizomatous plants. Rhizomes are considered underground stems, and are not roots and plants that naturally spread by rhizome often form carpets or thickets, these plants are great excellent choice for propagation by rhizome through cutting or division. The propagation of *Paris polyphylla* a perennial herb has been studied in the past by Raomai *et al.*, (2014a; 2014b) using immature zygotic embryos through direct somatic embryogenesis and thin cell culture. These methods on propagation however requires high level of expertise and are mostly confined to research studies unless a fine tuned friendly protocol is provided. The present study was thus focused on the use of rhizome as clonal planting material as seed requires two years for germination with less germination rate and thus does not provide an ideal choice of planting material.

During the study it was observed that the regenerative potential of *Paris polyphylla* from excised rhizomes were restricted to those rhizomes which have lateral buds, and those excised rhizomes without any budding were not able to give rise to any

plantlets and the excised rhizomes only develops roots or gets rotten. Excised rhizomes with developing buds were observed to be developing lateral buds and thus the potential of its further development and giving rise to plantlet is conspicuous. The excised rhizomes with already developed buds were successfully able to regenerate into individual plantlets and can be an efficient alternative to overcome the long term sexual regeneration in *Paris polyphylla*.

The matured rhizomes were observed to be developing new lateral buds giving rise to 1-6 new rhizome forming clumps. These lateral buds are mostly dormant and it is the terminal bud that develops into plantlet and then gives rise to a new terminal bud which will develop into plantlet for the next season. The lateral buds usually get covered with scales forming the inter-node and then develop new buds. This apical dominance in rhizomes was also reported in *Calamagrostis canadensis* (Powelson and Lieffers, 1991) and *Elytrigia repens* (Macintyre and Cessana, 1998) where the presence of the terminal bud or apical buds dominates over the lateral buds but some matured lateral branches were observed having the potential of plantlet regeneration while still attached with the parent plant thus forming thickets but most of the younger lateral branches were not able to develop plantlets.

The climate suitability model developed earlier as described in chapter 2 provides different prediction threshold for site selection and based on the model rhizomes were tested in the three selected sites. The response of the plants in the two sites gives a significant result of 87% in predicted high suitability areas and 76% in predicted moderately suitable areas, and confirming the model prediction ability predicted low suitable areas gives a very low response rate of 47% and thus does not has all the necessary adaphic condition to support the plant growth and regeneration. The plants response to flowering and seed formation observed in nature is quite low despite of high

seed production in each seed capsule ranging from 5 to 80, and during field survey it was observed that the maximum plant population in the sub-population was only 20 (Table 4.3) and seed formation in nature has a higher success percentage of 29% (Maximum) compared to the testing sites with a maximum of only 13%, this could be due to some physiological requirements by the seeds or climatic factors contributing to this low population and requires further intensive study. The rhizomes however, give a very significant result in terms of multiplication from lateral buds or branches. This ability of the matured rhizome to grow from division and giving rise to new lateral buds can compensate to the low sexual reproduction in nature. In an earlier study on *Paris polyphylla* (Jamir *et al.*, 2015) reported 49.33% from cut rhizomes and 75% in group rhizome fragmentation and in *Panax pseudoginsing* (Jamir *et al.*, 2016), rhizome cuttings has a response rate of 51.72% and similarly in the case of *Panax pseudoginsing* plant propagation in nature through seeds are quite low. This study also shows the importance of site/areas selection, *i.e.,* areas which can support the plant species with its required necessities to grow and regenerate can give a better success rate. The site selected based on the climate suitability model developed, allows the testing and introduction of the rhizome to different predicted threshold sites thereby giving a confirmation to the models prediction ability and the benefit of using climate suitability models. This will aid in re-introduction programmes as plants can be introduced in suitable areas and not in randomly selected sites which otherwise could result in loss of labour and economy, though initial factors such as sexual regeneration due to absence of pollinating agents may be bottlenecks but in perennial herbs like *Paris polyphylla* the vegetative propagation ability of the rhizome can be an alternative to overcome such sexual regeneration factors. The potential of the rhizomes to give rise to new plantlets from rhizome cuttings and the ability of the rhizome to give rise to new lateral buds from the mother rhizome can be

effectively and successfully employed for the production of clonal planting material for this highly exploited medicinal plant and thus can be an alternative propagation technique compensating to the low and complex sexual regeneration in nature.

Summary and Conclusion

The present study explores the use of an alternative means of propagation for the highly threatened medicinal plant *Paris polyphylla* as seed propagation in nature is low and its propagation in controlled environment has not witness any significant success result so far. During the study the regenerative potential of *Paris polyphylla* from excised rhizomes with lateral swellings or lateral bud primordia and well developed lateral buds was tested and they successfully develop into plantlets and thus can be an efficient alternative technique to overcome the long term sexual regeneration.

The effect of climate suitability on the growth and regeneration potential of *Paris polyphylla* was tested using a climate suitability model developed earlier in Chapter-2. The response of the plants in relation to the different prediction threshold sites clearly confirms the need for suitable climatic parameters for flowering and seed setting to occur, thus also validates the success of climate models to describe areas and sites for introducing economically important plants. This study can serve as a model study/work for future conservation programmes on re-introduction of propagated plants in suitable areas as random re-introduction of economically important plants can be an effort wasted. The present study has successful exploited the regenerative potential of rhizomes for the production of clonal planting material for *Paris polyphylla*.

Chapter - 5

Propagation of *Vanda bicolor*

The members of the most evolved family Orchidaceae with an estimate of about 10% of the flowerings plants (Dressler, 1981) representing 25,000-30,000 species of some 800 genera in the family (Deb and Imchen, 2008). No plant family has as many different flowers as the Orchidaceae family. These thousands of orchid species make up a fascinating group of flowering plants which is distinct from other flowering plants. Orchids got their name from the Greek word '*Orchis*' meaning '*testicle*' the word was first used by Theophrastus (372/371–287/286 B.C.) in his book '*De Historia Plan Tarun*' (The Natural History of Plants) is considered the father of 'Botany and Ecology'. Orchids are perennial flower with zygomorphic or irregular flowers with two basic growth types: Monopodial (one footed) (e.g., *Phalaenopsis* spp., *Renonthera* spp., *Vanda* spp, etc. having a main stem which continues to grow each year and Sympodial (many footed) (e.g., *Cattleya* spp., *Cymbidium* spp., *Dendrobium* spp. etc). The Plant produces a series of adjacent shoots which grow to a certain size, blooms, then stop growing to be replaced by the next growth.

Orchids are cosmopolitan in their distribution occurring in almost every habitat, except Antarctica and deserts. Majority are to be found in the tropics, most Asia, South

America and Central America. They are found above the Artic Circle in Southern Patagonia *Arachnites uniflora* and even on Macquarie Island, close to Antarctica and new species are being added every year. India is amongst the most distinct and diverse biogeographic regions of the world Housing 177 genera with 1195 orchid species (Singh, 2001) which was updated to 1,331 species of orchids in 186 genera (Misra, 2007) and new reports and discoveries being added each year. Of total about 400 species are endemic to India (Misra, 2007). The Himalayan, the North Eastern region and the peninsular regions are major Habitats in the country. About 250 species of Indian orchids are threatened (e.g., *Vanda coerulea* Griff. ex. Lindl.), and some like *Aphyllorchis gollani, Paphiopedilum charlesworthii, Pleione lagenaria* and *Vanda wightii* have probably already extinct in nature (Singh, 2001). The North-Eastern region of India houses more than 1000 orchid species belonging to some 711 genera and Nagaland with an area of 16579 Km^2 is a home to 396 species of orchids belonging to 92 genera (Deb and Imchen, 2008). All Orchid species are protected for the purposes of international commerce under CITES (Convention on International Trade in Endangered Species) as potentially threatened or endangered in their natural habitat except hybrids. Orchid exhibits an outstanding range of diversity in Size, Shape and Colour. Orchids occupy top position among all the angiosperms and are valued for cut flower production and as potted plants because of their longer lasting and exceptionally beautiful flowers which has lured man to exploit this nature's beauty for his own benefit. Summing up to the already prevailing anthropogenic activities like 'Jhum', forest fire, logging, destruction of habitat due to urbanization etc, excessive collection for horticultural trade or for medicinal purpose has formed a bottleneck to the path of orchid conservation as orchid trade in this region is usually out sourced from the wild. Adequate measures if not taken up the probability of more species getting extinct from the wild is rather high and as orchid

propagation in nature is very low despite a single orchid producing millions of seed only few germinates and survives in natural conditions. The present trend calls for urgent intervention and measures to reduce the rampant exploitation of nature gift of beauty and micro propagation provides an efficient and viable tool for mass propagation of endangered orchid species.

Orchid micropropagation techniques has immensely advanced in the recent years but some problem like transplantation to field limits the survival of orchid's especially in epiphytic orchids. In culture condition optimum environment is provided artificially for the growth and regeneration of the plants however when the plantlets are transferred to *ex vitro* environment their ability to adjust to the harsh natural environment greatly reduces the survival rate. The ability of the plantlets to form new roots and leaf on a new substratum in natural environment reduces with the reduced minerals and organic supplements and climatic conditions in the new environment to which they are exposed. Thus transplantation remains a bottleneck in many orchids as substantial numbers of plantlets are unable to survive in the natural environment (Chugh *et al.*, 2009). The new natural environment with low humidity and altering temperature and light conditions along with the microbial infection in contrast to the *in vitro* conditions. The objective of micropropagation is the successful transfer and establishment of the cultured plantlets in the natural environment (Hazarika, 2003), and several workers has developed hardening techniques using chips of bricks, charcoal and decayed wood as substratum (Deb and Imchen, 2010), brick and charcoal mulched with moss (Kishor *et al.,* 2006), barks coconut coir and wood shavings (Franco *et al.,* 2007). The present study was undertaken for development of *in vitro* propagation protocols of *Vanda bicolor,* a commercially important vulnerable orchid species for mass propagation and establishment of regenerates in nature.

Materials and Methods

Plant Material Collection

Plants were collected during field trips to different parts of Nagaland after obtaining proper permission from concern authority and maintained in green house for flowering and seed pod formation for culture initiation.

Seeds: Immature green pods ~4-8 months after pollination (MAP) are cut with sharp blade and brought to the laboratory and is cleansed off with tap water and stored in room temperature during the months between July-September from the mother plants collected during field survey and maintained in greenhouse.

Leaf: For foliar explants, leaves from *in vitro* sources *i.e.,* from the plantlets raised from the seed culture were collected after about ~5-6 weeks in regeneration medium, those plantlets which have fully differentiated leaves and roots taken out of the culture tubes and the excised leaves are cleansed off for any agar residues and used for the study.

Roots: For aerial roots also, *in vitro* grown explants i.e., from the well differentiated plantlets raised from the seed culture of about 5-6 wk old after sub-culturing in regeneration media were selected as explants. Those plantlets which have fully differentiated leaves and roots taken out of the culture tubes and the excised roots are cleansed off for any agar residues and used for the experimental purpose.

Surface Cleaning and Sterilization

Seeds: The green pods are surface cleansed by washing in running water with detergent (Labolene 1:100 v/v) and finally rinsed with running tap water. The seed pods are taken to the laminar flow chamber and again rinsed with sterile water 4-5 times; the pods are surface sterilized with mercuric chloride ($HgCl_2$ 0.2%, w/v) for 5 min and rinsed with sterile water 4-5 times followed by dipping in ethanol (70%, v/v) and washed with sterile

water (4-5 times). The pods were then dipped in ethanol and flamed before the seeds pods were split opened and seeds were scooped out and cultured on germination medium.

Foliar and aerial roots **-** Foliar and aerial roots explants were obtained from *in vitro* sources and only those without any infections or contaminations were chosen for the purpose. Thus the explants are cultured without any sterilization after cleansing of the agar residues with sterile soft brush.

Tissue Culture Medium

For the present study MS medium (Murashige and Skoog, 1962) was used as nutrient basal medium. Nutrient medium was supplemented with sucrose (3%, w/v), different quality and quantity of plant growth regulators *viz.* α-naphthalene acetic acid (NAA) and N^6-benzyl adenine (BA) (0-9 µM) singly or in combination. Nutrient medium was gelled with agar (0.8%, w/v) and the *p*H was adjusted to 5.6 using 0.1N NaOH and 0.1N HCl. About 15 ml medium was dispensed in borosilicate test tubes (150 x 25 mm), and 30 ml in culture bottles (70 mm diameter, 400 ml). The medium was autoclaved at 1.05 Kg cm^{-2} pressure and at 121^oC for 20 min.

Initiation of Cultures

Seeds: The immature seeds/embryos of various developmental age was scooped out from the sterilized green pods with the help of sterilized scalpel blade and cultured on fortified MS medium conjunct with sucrose (3%, w/v) and different concentrations of NAA and BA (0-9 µM either singly or in combination). For each treatment 10 test tubes were maintained.

Foliar Explants: Leaves from *in vitro* raised plantlets were carefully taken out in the laminar flow cabinet and removed the traces of agar residues with soft sterilized brush and sterilized water. The foliar explants are cultured on agar based MS medium fortified with sucrose (3%) and different combinations of NAA and BA (0-9 µM, singly and in

combination). Explants were placed in different orientations to study the effect of orientation. For each treatment 20 explants were maintained.

Aerial Roots: Aerial roots from *in vitro* raised plantlets were carefully taken out in the laminar flow cabinet and were cleansed off for agar residues with soft sterilized brush and sterilized water and the explants are excised into two equal halved horizontally or vertically. Explants were cultured on agar based MS medium fortified with 3% sucrose and different combination of NAA and BA (0-9 µM, singly or in combination).

Culture Conditions: All the cultures were maintained in diffused light (20 µmol $m^{-2}s^{-1}$) and full light (40 µmol $m^{-2}s^{-1}$) provided with white fluorescent light at 12/12 h light/ dark photo cycles and at 25±2^{o}C temperature. Cultures were sub-cultured at 5-6 wk interval unless mentioned otherwise.

The PLBs and shoots developed from the germinated seeds, cultured aerial roots, and foliar explants were maintained for another two subcultures on optimum initiation culture for further growth and differentiation.

Experimental Design and Statistical Analysis

The present study was carried out to optimize culture protocol for mass multiplication and regeneration of *Vanda bicolor*. In the present study completely randomized experimental design were followed. All the experiments were repeated five times and data represents the mean of five replicates. Data were collected at weekly interval and analyzed for statistical significance using statistical analysis software SPSS version 17.01.

Plant Regeneration and Mass Multiplication

The PLBs and shoots produced from different explants on initiation medium were separated from the clumps and cultured on MS medium fortified with sucrose (3%) and different combination of NAA and BA (0-9 µM, either singly or in combination) alone

and in combination and sub-cultured at 5-6 wk for plant regeneration and culture proliferation. Cultures were monitored at regular interval for shoot and root development. A set of advanced stage PLBs are also inoculated in MS medium supplemented with different concentration of activated charcoal (0-0.9% w/v) to study its influence on culture proliferation and regeneration. The well rooted plantlets from the regenerated medium were selected for *in vitro* hardening.

Hardening and Transplantation to Potting Mix

The regenerated plantlets with well developed roots are transferred for hardening following the technique given by Deb and Imchen (2010). For the purpose, $1/10^{th}$ strength MS salt solution with full strength organic components but without sucrose and PGRs was used. This liquid solution was used in the cultured vials containing a matrix of charcoal pieces, brick pieces, crushed decayed wood and moss in the ratio 1:1:1:1 The cultures were maintained in diffused light (20 µmol $m^{-2}s^{-1}$) and full light (40 µmol $m^{-2}s^{-1}$) at 12/12 hour light and dark photo cycles at 25±2^{o}C for 5-6 wk. and transferred to community potting mix (charcoal pieces, brick pieces and sand in the ratio 1:1:1). The potted plants are maintained in poly house ca. 70% shading and well hardened plants are transferred to natural environment.

Preparation of Substrata for Hardening and Potting Mix

For effective cost reduction the substratum for hardening and potting mix was prepared using locally available materials. The substratum consists of a matrix of charcoal pieces, brick pieces, moss and crushed decayed wood in the ration 1:1:1:1. For hardening and for potting mix sand was added to the above combination. The materials are collected and washed thoroughly in tap water and sundried, the well dried charcoal pieces, brick pieces, moss and crushed decayed wood are then autoclaved at 121^{o}C for 20 minutes and

at 1.05 Kg cm^{-2} pressure. The cleansed and autoclaved materials are mixed in the ratio of 1:1 and used as substrata for hardening and community potting mix.

Development of New Hardening Procedure

Besides above technique, an attempt was made to develop a new hardening technique. In this endeavour dried decaying wood with high porosity as well as burnt wood pieces with charcoal were collected from forest and sun dried, the dried woods are then cut into small pieces (7-8 cm length) and washed thoroughly in running tap water, the wood pieces are then soaked in Bavistin (5% w/v) for one night and washed off with sterile water (4-5 times) and autoclaved at 121^{o}C for 20 min at 1.05 Kg cm^{-2} pressure. After autoclaving the wood pieces were boiled in 1/10th strength of MS salt solution without any organic nutrients and PGRs to retain the MS medium in the porous dried wood. The wood pieces were then put in jam bottles (culture vials) with filter paper soaked in MS Medium (1/10th MS salt solution) and further autoclaved at 121^{o}C for 20 min. Fully differentiated plantlets raised *in vitro* from different explants were transferred to the wood pieces. The fully differentiated plantlets are cleansed using a thin sterile brush to remove traces of agar and are slowly set on the wood pieces in laminar flow chamber. The plantlets are then maintained under normal laboratory conditions (25±2^{o}C and 12/12 h light and dark photoperiod). Cultures were observed for development of velamen and velamenous root attachment to the wood pieces. After the roots were attached to the wood pieces the cap of the culture vials were removed and kept open and maintained for ~4 wk. Regular checking was done to keep the cultures from drying up and watered (supplemented with 0-1% sucrose) using a dropper. Well established plantlets with firm velamenous attachment to the wood pieces are directly transferred to greenhouse and watering was done at regular intervals using a dropper.

Table - 5.1: Effect of green pod age/developmental age of immature embryos of *Vanda bicolor* on *in vitro* germination

Seed pod age	Time for germination	Germination rate (%) (±)*	Type of response
4	-	-	-
5	76	30 ±0.28	Delayed germination
6	48	64 ±0.26	Healthy green PLBs
7	40	85 ±0.27	Healthy Green PLBs
8	42	68 ±0.26	Healthy Green PLBs

On MS media with sucrose (3% w/v) treated with 3 µM NAA and BA.
* Data represent a mean of 5 replicates ±SE.

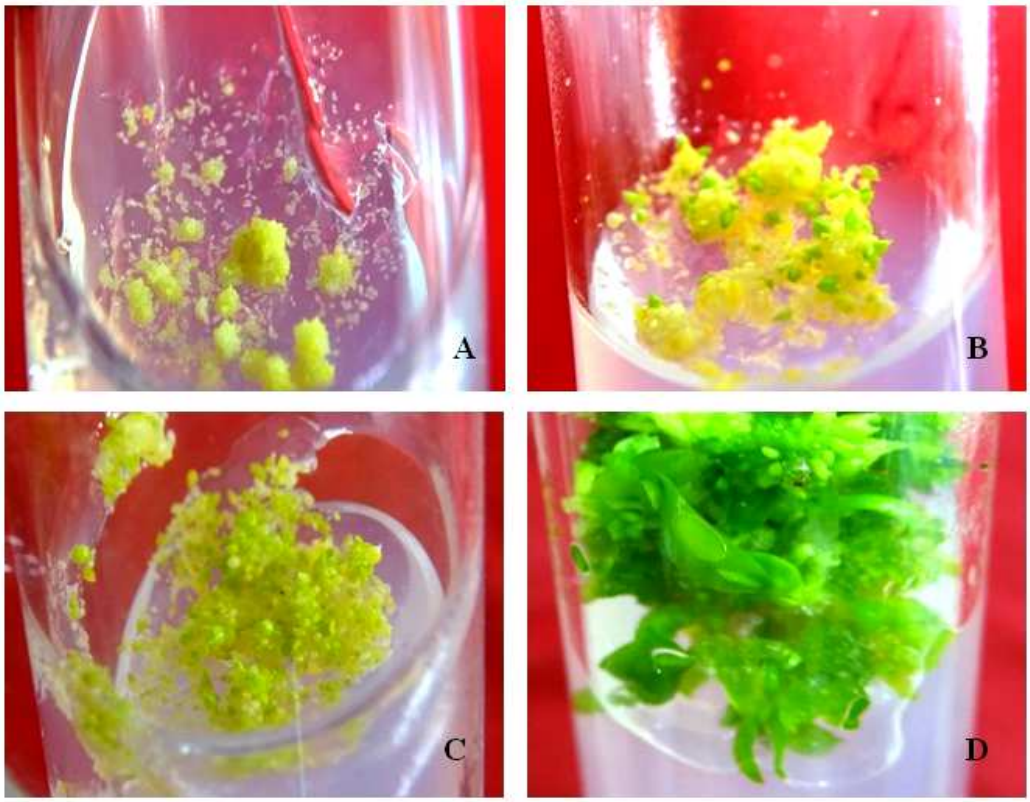


Figure - 5.1

Figure - 5.1: Different stages of seed germination of *Vanda bicolor*. A. Swelling of seeds showing the first sign of germination, B. & C. Development of PLBs from germinating seeds, D. Plantlets formed from germinating seeds.

Table - 5.2: Effect of quality and quantity of PGRs on *in vitro* germination of immature seeds of *Vanda bicolor*

PGRs Conc. (µM)		Time for germination (days)	Time for PLBs formation (days)	% response ±SE*	Type of response
NAA	BA				
0	0	40	52	56 ±0.37	Small unhealthy PLBs formed unhealthy
3	0	40	50	72 ±0.31	Small green PLBs formed unhealthy
6	0	30	48	68 ±0.37	Small unhealthy PLBs low germination rate
9	0	30	48	64 ±0.24	Small unhealthy PLBs formed late germination
0	3	35	50	60 ±0.24	Small unhealthy PLBs
0	6	30	48	64 ±0.31	Small unhealthy PLBs
0	9	30	50	78 ±0.37	Small unhealthy PLBs
3	3	25	35	88 ±0.24	Healthy PLBs followed by shoots formation
3	6	35	48	84 ±0.20	Healthy green PLBs
3	9	25	35	76 ±0.20	Healthy green PLBs
6	3	25	48	84 ±0.24	Green PLBs and shoot formation
6	6	25	35	84 ±.20	As above
6	9	40	50	80 ±0.32	Green PLBs and shoot formation
9	3	30	48	76 ±0.20	Green PLBs and shoot formation
9	6	30	48	76 ±0.20	Green PLBs and shoot formation
9	9	40	52	68 ±0.24	Unhealthy PLBs formed

On MS media with sucrose (3% w/v) treated with NAA and BA

* Data represent a mean of 5 replicates ±SE.

Results

Seed Germination: The immature seeds (4-8 MAP) were cultured on MS medium fortified with sucrose (3%) containing different combination of PGRs (NAA+BA, 0-9 µM) alone and in combination. Seeds from younger green pods (4 MAP) failed to register germination. With increase in green pod age, germination improved. Of the different developmental age, seeds of 7 MAP registered optimum germination (85%) under given condition after 40 days of culture initiation (**Table - 5.1**). The first sign of seed germination was observed by the seeds turning into yellowish colour after 25 days, and PLBs were observed after 35 days (**Figure - 5.1 A, B, C and D**). Among the different concentrations of PGRs a combination of NAA and BA gives a better germination rate against the singly treatment of BA and NAA (**Table – 5.2**). Of the different combinations of PGRs 3 µM NAA + 3 µM BA with 88% germination rate followed by 3 µM NAA + 6 µM BA, 6 µM NAA + 3 µM BA and 6 µM NAA + 6 µM BA with 84 % (**Table - 5.2**). Combinations of PGRs 3 µM NAA + 3 µM BA resulted in better germination with subsequent development of healthy PLBs. Incorporation of PGRs in the germination medium was prerequisite for germination of immature seeds and on PGR controlled medium with no PGRs seed germination was not observed. Of the two PGRs tested for seed germination, early seed germination was observed on medium fortified with higher concentration of BA. Germination response was significantly different in those culture medium treated with a combination of NAA and BA and those culture mediums with treated with NAA alone. Better germination and differentiation into PLBs was better on nutrient medium enriched with NAA and BA in combination.

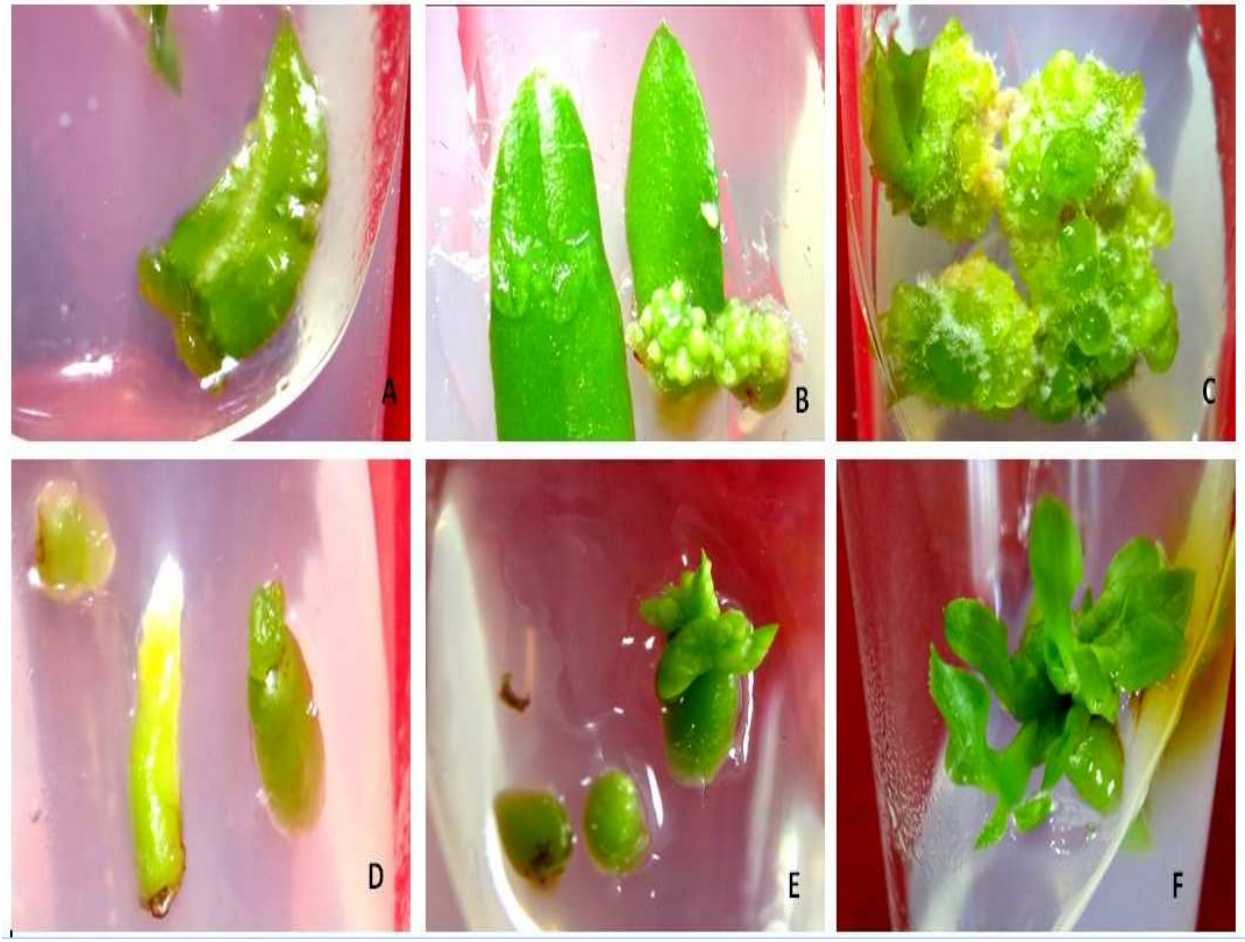


Figure - 5.2

Figure - 5.2: Different stages of morphogenesis from foliar explants (A-C); aerial root (D-F). A. Cultured leaf showing swelling at the cut end, B. Formation of PLBs, C. PLBs formed throughout the leaf, D. Cultured roots showing the swelling of roots as first sign of morphogenesis, E. Shoot buds formation from the cultured roots, F. Plantlets formed from the roots.

Table - 5.3: Effect of PGRs on *in vitro* morphogenetic response of foliar explants from *in vitro* source

PGRs Conc. (µM)		Time for initial response (d)	Time for PLSs formation (d)	% response ±SE*	Type of response
NAA	BA				
0	0	-	-	-	No response
0	3	20	90	48 ±0.30	Unhealthy PLBs
0	6	20	90	60 ±0.25	Unhealthy PLBs
0	9	-	-	-	Tissue thickening but no PLBs initiation
3	0	-	-	-	Tissue thickening but no PLBs initiation
6	0	-	-	-	Tissue thickening but no PLBs initiation
9	0	-	-	-	Tissue thickening but no PLBs initiation
3	3	16	36	86 ±0.25	Prominent swelling at leaf base leading to healthy PLBs initiation
6	3	20	45	68 ±0.24	Swelling at leaf base leading to PLBs initiation
9	3	16	36	74 ±0.28	Swelling at leaf base leading to healthy PLBs initiation
3	6	16	36	78 ±0.20	Swelling at leaf base leading to healthy PLBs initiation
6	6	20	45	60 ±0.37	Swelling at leaf base leading to PLBs initiation
9	6	-	-	-	Tissue thickened
3	9	-	-	-	No response
6	9	25	-	50 ±0.28	Slight swelling but no PLBs initiation
9	9	25	-	38 ±0.31	Slight swelling but no PLBs initiation

On MS medium with sucrose (3% w/v).

* Data represent a mean of 5 replicates ±SE.

Table - 5.4: Effect of PGRs on *in vitro* morphogenetic response of aerial roots from *in vitro* source

PGR Conc. (µM)		Time for initial response (d)	Time for PLBs formation (d)	% response ±SE*	Type of response
BA	NAA				
0	0	-		-	No response
3	0	40	-	-	Swelling at root tips but no PLBs formation
6	0	36	-	-	Swelling at root tips but not PLB initiation
9	0	40	-	-	Swelling at root tips and formation of hair like structures but no PLB initiation
0	3	40	-	-	Swelling at root tips and formation of hair like structures but no PLB initiation
0	6	40	-	-	As above
0	9	35	56	50 ±0.37	Root tips swollen leading to formation of few unhealthy PLBs
3	3	21	40	74 ±0.24	Swelling at root tips leading to formation of healthy multiple PLBs
3	6	35	60	46 ±0.31	Swelling at root tips and formation of hair like structures leading to formation of few unhealthy PLBs
3	9	40	60	68 ±0.20	formation of hair like structures leading to formation of few unhealthy PLBs
6	3	30	43	74 ±0.28	Swelling at root tips leading to direct shoot generation
6	6	35	60	26 ±0.37	Swelling at root tips leading to direct shoot generation
6	9	-	-	-	Tissue thickened but no PLBs formation
9	3	40	-	-	Swelling at root tips leading to initiation of few unhealthy PLBs
9	6	35	-	-	Swelling at root tips and formation of hair like structures but no PLB initiation
9	9	35	-	-	Swelling at root tips and formation of hair like structures but no PLB initiation

On MS medium with sucrose (3% w/v).

* Data represent a mean of 5 replicates ±SE.

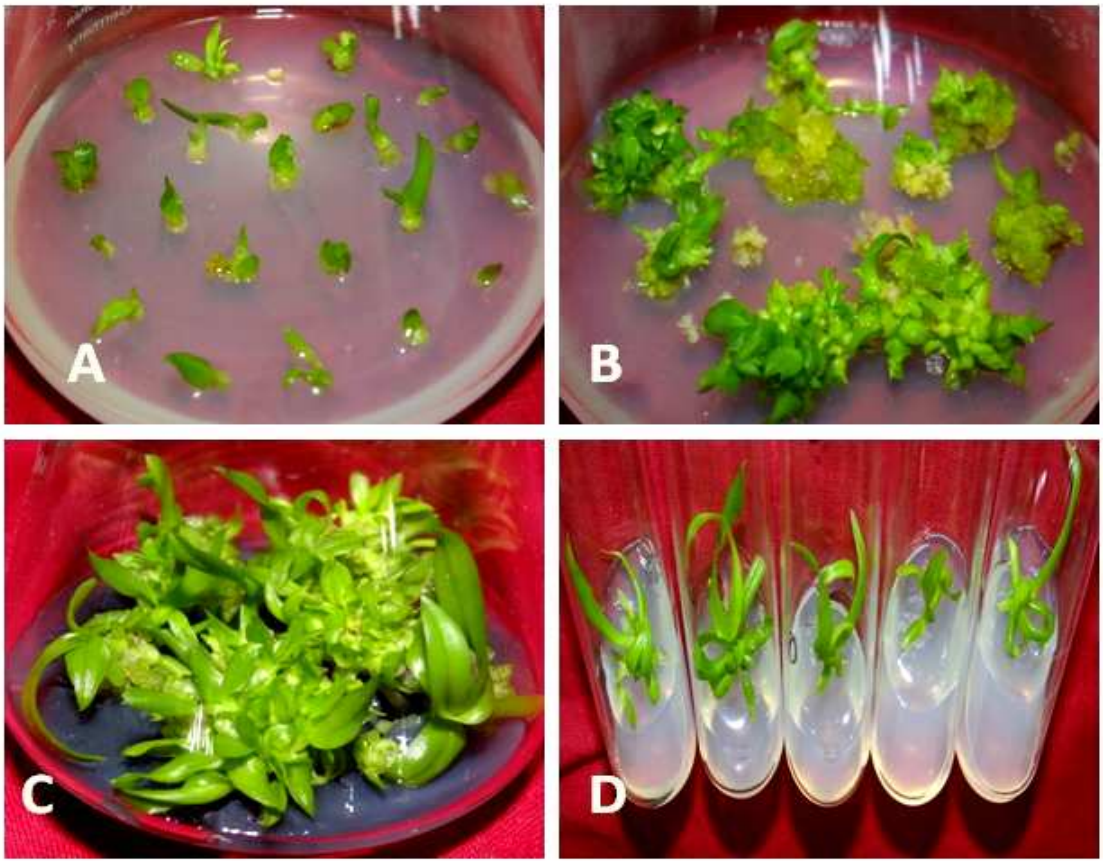


Figure - 5.3

Figure - 5.3: A. Advanced stage PLBs in regeneration medium, B. Proliferation of culture in regeneration medium, C. Regeneration medium fortified with AC showing healthy plant growth, D. Regenerated plantlets with healthy roots.

Leaf Culture

From the cultured foliar explants the first sign of response was observed as leaf curling, tissue thickening followed by swelling of leaf. First sign of response initiated from the basal part after ~16 days of culture and PLBs developed after 36 days of culture **(Figure - 5.2 A, B, C**). Of the different concentrations of PGRs, a combined treatment of NAA and BA supported better morphogenetic response over NAA or BA alone (**Table - 5.3**). Among the different combinations of PGRs, 3 µM NAA + 3 µM BA with 86% and 3 µM NAA + 6 µM BA with 78% response rate supports better PLBs formation. PLBs initiation was observed better in those cultures with a combined treatment of NAA and BA than those culture medium treated with NAA and BA alone.

***Root culture*:** In root culture the first sign was observed as tissue thickening and swelling of the roots after 21 days, and PLBs were observed after 40 days of culture (**Figure - 5.2 D, E, F**). Direct shoot generation was also observed in cultures with a combined treatment of NAA and BA. Of the different concentrations of PGRs, a combination of NAA and BA registered better result than NAA or BA alone (**Table - 5.4**). Among the different combinations of PGRs 3 µM NAA + 3 µM BA gives a better PLBs initiation with 74% response followed by 3 µM NAA +9 µM BA with 68% but does not support healthy PLBs initiation.

Regeneration and Mass Multiplication

Advanced stage PLBs (**Figure 5.3** A) were transferred on MS medium enriched with NAA and BA in alone or in combination. The present study showed that a combined treatment of NAA and BA resulted in better regeneration of plantlets over single treatment of PGRs (**Figure 5.1 D, 5.3 B)**. Simultaneous shoot and root development was observed in those culture mediums treated with NAA and BA in combination. Of the different combinations 6 µM NAA + 3 µM BA, 6 µM NA+ 6 µM BA supported better

rooting and healthy shoot development with 86% of the culture responding to both the treatments (**Figure 5.3 D)**. Simultaneous root and shoot development with PLBs initiation was achieved in combined PGRs treatment (**Table - 5.5**). Culture medium treated with NAA and BA alone did not support regeneration of plantlets and resulted in stunted growth of plantlets and their subsequent degeneration. The nutrient medium was conjunct with NAA and BA and AC (0.3-0.9%) to study the effect on mass multiplication and regeneration. In each test tube a single fully differentiated plantlet was sub-cultured on MS Medium supplemented with AC. A concentration of 0.6% (w/v) AC supported healthier plantlets with dark green shoots and healthy rooting. Secondary PLBs formation was also observed on AC rich medium (**Table -5.6, Figure – 5.3 C**).

Hardening and Transfer to Potting Mix

Plantlets with well developed roots and leaves are transferred for hardening following the technique described by Deb and Imchen (2010). Culture vials were prepared by putting a matrix of moss, charcoal, brick pieces and decayed wood/moss in the ratio 1:1:1. The matrix was soaked with $1/10^{th}$ strength MS inorganic salts solution without sucrose and PGRs. The plantlets were taken out from the culture medium and washed to remove agar residues and 2 plantlets were cultured in each culture vials as mentioned above and maintained in normal laboratory conditions for 90 days (**Figure - 5.4 A**). It was observed that during the *in vitro* hardening process the roots of the hardened plantlets adhered to the brick or charcoal pieces (**Figure - 5.4 B**). The hardened plantlets were transferred to potting mix which is composed of charcoal pieces, brick pieces and moss in the ration 1:1:1. The plantlets are maintained in greenhouse and nourished with $1/10^{th}$ liquid MS medium at regular interval. About 90% transplants survived after 8 wk of transfer and the established transplants were transferred to natural habitat subsequently.

Table - 5.5: Effect of PGR's on morphogenetic response on plant regeneration and mass multiplication

PGR Conc. (µM)		% response ±SE*	No. of shoots/roots developed per explant	Type of response
NAA	**BA**			
0	0	44 ±0.25	3/2	Plant growth stunted
0	3	76 ±0.25	3/3	Moderate growth
0	6	68 ±0.47	4/3	Moderate growth and poor rooting
0	9	76 ±0.28	3/2	Moderate growth and poor rooting
3	0	44 ±0.25	3/3	Healthy shoots but poor shooting
6	0	54 ±0.25	3/3	Healthy shoots but poor shooting
9	0	60 ±0.31	3/4	Healthy shoots but poor shooting
3	3	80 ±0.30	7/4	Healthy plantlets with good rooting and Multiple PLB's formation
6	3	86 ±0.25	6/3	Healthy shoots but Poor rooting
9	3	78 ±0.25	7/4	Multiple PLB's formation and healthy shoots with well rooted plantlets
3	6	84 ±0.25	5/4	Multiple PLB's formation and well rooted plantlets
6	6	86 ±0.25	4/3	Moderate growth and rooting
9	6	74 ±0.41	4/4	Growth stunted
3	9	72 ±0.25	3/4	Growth stunted
6	9	76 ±0.24	3/4	Growth stunted
9	9	68 ±0.25	4/3	Growth stunted

On MS media with sucrose (3%).
* Data represent a mean of 5 replicates ±SE.

Table - 5.6: Effect of activated charcoal on morphogenetic response on plant regeneration and mass multiplication of *Vanda bicolor*

AC Conc. (%)	No. of shoots/roots developed per subculture	Type of response
0.3%	3-6 shoots with 2-4 aerial roots	Healthy plantlets with deep green leaves and well rooted
0.6%	3-8shoots with 2-5 aerial roots	Healthy plantlets with deep green leaves and excellent rooting
0.9%	2-4 shoots with 2-4 aerial roots	Healthy plantlets with deep green leaves and well rooted

On MS media with sucrose (3% w/v), 3 µM each of NAA and BA.

Figure - 5.4

Figure - 5.4: A. Plantlet in hardening matrix, B. Hardened plantlets showing adhering of roots to charcoal and brick pieces, C. Transplants established in the potting mix, D. Regenerate transferred in the natural habitat.

Figure - 5.5

Figure - 5.5: Different stages new hardening technique. A. Plantlets set on wood pieces inside culture vials, B. Plantlets with well developed velamen adhered to the wood pieces, C. Plantlets set on burnt wood pieces transferred to greenhouse and D. Well established plant in greenhouse.

New Hardening Technique

Plantlets with fully differentiated *in vitro* raised plantlets from different explants were first sub-cultured in reduced strength MS medium ($1/10^{th}$) for 30 days followed by set on the processed and sterilized dead wood pieces and burnt wood pieces as described in materials and methods (**Figure – 5.4 A**). After about 60 days of transfer velamenous roots starting adhering to the wood pieces (**Figure - 5.5 B**). Plantlets set on burnt wood pieces established better by strong attachment of velamen compared to the dead wood pieces (**Figure - 5.5 C**). The cultures maintained under normal laboratory conditions for three months exhibited a mixed response. About 70% transplants exhibited adhering of roots to wood substratum. These plants could be transferred to poly house/green house directly without any additional processing like potting etc. The transplants in the poly house registered over 90% survival accompanied with healthy plant growth. Well hardened and established plantlets were transferred to natural forest and set on tree bark (**Figure 5.5 D**). The new hardening technique gives a high field transfer survival rate and the only limitation lies during the development of velamenous attachment of the plant roots to the wood pieces as some plantlets takes longer time to establish on the wood pieces, which otherwise proves to be an efficient hardening technique and does not require transplantation of the plants to community potting mix and requires further investigation.

Discussions

The role of orchid germination is of immense importance for successful orchid conservation strategies. However, in its initial stages bottlenecks related to orchid germination like orchid seed fungal association has been a major factor for *in vitro* germination of orchid seeds. Records for successful germination of orchid seed germination goes back to 1903 when Bernard tried to germinate *Bletilla Hyacintha* seeds

and reported a very less optimum level of 2%, the knowledge in orchid aysmbiotic germination like the role of sucrose came to light when Knudson (1919) germinate seeds of *Cattleya labiata* crossed with *Cattleya aurea* on carrot and beet concoctions and thus achieved a breakthrough in asymbiotic orchid seed germination. The seeds germinated and develop seedlings on both media which convinced him that orchid seeds can germinate without fungus (Arditti, 1990). The discovery of PRGs by Went (1926) was another important breakthrough in the history orchid asymbiotic seed germination leading to discovery of other plant hormones and their significant influence on orchid germination. Orchid asymbiotic germination needs a medium which supplies all organic and inorganic supplements as well as plant growth regulators and sugar needed by the cultured embryos to germinate and grow, different orchid genera and species may respond in different ways to different set of media and plant growth regulators. Recent studies has shown successful asymbiotic seed germination in different set of PGRs and growth medium in different species of orchids like *Cymbidium iridoides* (Deb and Pongener, 2013) where 95% germination was reported on agar based MS medium, 90% on betel-nut coir, 85% on foam and 50% on forest litter, with optimum response achieving at media supplemented with PGRs combination of 3µM NAA+6 µM BA and thus developing an efficient cost effective protocol for asymbiotic orchid seed germination; *Malaxis acuminata* (Arenmongla and Deb, 2012) reported 85% on agar gelled MS medium supplemented with 4 µM NAA.

In the present study with *Vanda bicolor* Griff a rare Monopodial orchid, asymbiotic seed germination was initiated on agar based MS medium from immature seed of 7 MAP. The initial sign of response was observed as a change of colour (turning yellow) and nodular swelling of seeds which differentiates into PLBs within a period of 25-35 days. Optimum seed germination and initiation of PLBs was observed in cultures

treated with 3 µm NA + 3 µm BA, giving a better gemination with full vigour achieving 88% germination using 7 months old seedpods. Cultures without any PGRs showed delayed or no germination. Single treatment of both the PGR did not support optimum germination and growth of the embryos though signs of early germination were observed in cultures treated with BA. A combined treatment of both the PGRs is observed to give optimum germination rate for the selected orchid species. These results are in agreement with earlier works on asymbiotic orchid seed germination (Temjensangba and Deb, 2006; Arenmongla and Deb, 2012; Deb and Pongener, 2013) where significant difference in germination were observed in cultures treated with PRGs (NAA+BA) and those without any treatment. However, seeds germination within 40 - 45 days in Vacin and Went medium with 1.0 mg/l BAP + 0.5 mg/l NAA + 2% sucrose + 2 g/l peptone has also been reported in related species like *Vanda teres* (Sinha and Roy, 2004).

Cultures initiated from foliar explants successfully developed into PLBs. The initial sign of response was observed as curling and swelling of the leaf tissue this is followed by swelling at the basal end of the leaf which differentiates into PLBs after 36 days. All meristematic activity was observed only at the base of the leaf where the explants are excised and the orientation of explants in the basal media does not show any significance difference in PLBs initiation. Optimum result was observed in a combined treatment of PGRs (3 µM NAA + 3 µM BA). PLBs initiation was observed to be better in cultures with a combination of NAA and BA with about 86% response with high PLBs initiation. These results of shoot culture with treatment of PGRs showed a similar pattern on earlier observation made by Vij and Pathak (1990); Temjensangba and Deb (2005); Deb and Temjensangba (2007a); Deb and Sungkumlong (2010); Paudel and Pant (2012); Kaur and Bhutani (2009) where treatment of PGRs shows significant increase in response rate. In other related species like *Vanda coerulea* rapid PLBs initiation was achieved

through shoot tips of mature plants, shoot tips and leaf base on Mitra *et al.*, medium treated with 8.8 µM BA and 4.1 µM NAA (Seeni and Latha, 2000).

Aerial root explants from *in vitro* source started PLBs formation after ~40 days of culture initiation while, the first sign of response was seen as tissue thickening and swelling of the roots only after 21 days of culture. All meristematic activity was initiated from the root tips. Optimum initiation was observed in cultures treated with 3 µM BA + 3 µm NAA. Optimum PLBs initiation was observed in cultures with a combination of NAA and BA. Similar results were observed in *Arachnis labrosa* and *Cleisostoma racemiferum* (Deb and Temgensangba, 2005, 2006), in *Vanill planifolia* (Philip and Nainer, 1986) PLBs initiation from root explants using IAA and kinetin as growth regulators has also been reported.

The differentiated PLBs resulted from different explants were separated and sub-cultured on MS medium enriched with NAA and BA either singly or in combination. Optimum growth and regeneration with simultaneous shoot and root development was observed in those cultures treated with NAA and BA in combination. Optimum culture condition for regeneration was observed in combined treatment of 3 µM NAA + 6 µM BA with healthy shoot and root development. Culture medium enriched with higher concentrations of BA singly did not support healthy regeneration while, simultaneous root and shoot development with PLBs initiation was observed in combined PGRs treatment. Culture treated with NAA and BA alone results in stunted growth of plantlets and their subsequent degeneration. Cultures without PGRs exhibited stunted growth and slowly degenerate. Similar pattern of response were observed in earlier studies with other orchid species where presence of both NAA and BA promoted healthy shoot formation and multiple rooting (Arenmongla and Deb, 2012; Deb and Pongener, 2013).

The role of activated charcoal has been proved to be an important tool for plant tissue culture for controlling problems related phenolic extracts as activated charcoal acts as an adsorbent of inhibiting exudates or substances in the culture medium and as stimulatory agent in shoot and root induction (Thomas, 2008). Activated charcoal has been used in orchid tissue culture to negate browning and promotes shoot induction in *Cymbidium forrestii* (Paek and Yeung, 1991), promotes shoot formation and rooting in *Anoecthochilus forosanus* (Ket *et al.,* 2004), while, Chen *et al.* (2005) observed 90% rooting in *Cymbidium faberi*. In the present study addition of AC (0.3-0.9% w/v) in the regeneration medium (MS medium with 3 µM NAA + 3 µM BA) resulted in healthy dark green shoot accompanied by healthy rooting. Besides these, AC enriched medium also supports secondary PLBs formation. Of the different concentrations of AC tested a concentration of 0.6% (w/v) was observed to be the ideal concentration for regeneration.

Hardening is a very important part of tissue culture to ensure the survival of the plantlets in natural conditions. During the present investigation hardening of the plantlets was done following Deb and Imchen (2010). Highly reduced strength MS medium strength ($1/10^{th}$) of MS mineral salts without organic components, sucrose and PGRs in a matrix of moss, charcoal, brick pieces and decayed wood/moss in the ratio 1:1:1. Well developed plantlets are washed off for any agar residues and in each culture vials 2-3 plantlets are cultured and maintained in normal laboratory conditions for 3 months. It was observed that during this period the roots develop velamenous attachment to the charcoal and brick pieces. These plantlets are later transferred to CPM and maintained in green house with 90% survival rate.

To enhance the performance of the plantlets in their natural condition a new way of hardening was devised, in this method dead and decayed wood piece or burnt wood pieces are sterilized and soaked in highly reduced strength MS medium devoid of any

PGRs and sucrose. Well developed plantlets are then set on the wood pieces and these plantlets develop velamenous attachment within a period of 60-90 days. It was also observed that the velamenous attachment was better with burnt wood pieces than decayed wood pieces this could be due to the presence of charcoal which enhances the growth and development of the plantlets. The cultures maintained under normal laboratory conditions for three months shows a mix response during the development of velamenous attachment with only 70% of the plantlets adhering to the wood substratum however the advantage of this techniques lies during the transfer of the plantlets to the green house with high survival rate of 90% which is the main hurdle for tissue culturist to establish *in vitro* raised plants to field condition. The roots already having developed velamenous attachment to the wood pieces will not be disturbed and their establishment in natural condition is much enhanced. Well established and hardened are later shifted to green house or to natural forest and set on tree bark. The new hardening technique gives a high field transfer survival rate and the only limitation lies during the development of velamenous attachment of the plant roots to the wood pieces as some plantlets takes longer time to establish on the wood pieces, which otherwise proves to be an efficient hardening technique and does not require transplantation of the plants to community potting mix.

Summery and Conclusion

For orchid micropropagation seeds provide an ideal choice, as orchid seed pods contains large numbers of microscopic seeds without endosperm and micropropagation techniques provides all the necessary conditions for the germination, growth and development of these seeds. In the present study immature seeds of ~ 7 MAP germinated and formed PLBs where ~85% seeds germinated on MS medium fortified with sucrose (3%) and NAA + BA (3 µM each in combination). The germinated seeds formed PLBs

after 36 days of culture initiation. Rooted plants developed from advanced stage PLBs on MS medium fortified with 3 µM each of NAA and BA and AC (0.6%). Plantlets were hardened following Deb and Imchen (2010) achieved survival percentage of 90% and the well established plantlets are transferred to natural habitat. Further during the present study a new cost effective and efficient hardening technique is developed which will open up new route for hardening of tissue culture plant. The present study successfully developed the *in vitro* propagation technique of *Vanda bicolor,* a commercially important orchid and will help in propagation and conservation of this threatened species.

Chapter – 6

Summary

Existing agricultural practices, exploitation of natural resources and anthropogenic activities has reduced the population size of many important plant species. Environmental conservation policies and laws are not effective as more emphasis is on traditional practice of resources utilization. Thus the need of modern GIS technologies and tissue culture technique for species recovery and re-introduction can be a promising solution. The present study explores to bring out strategies for conservation and propagation of two economically important plant species *viz*. *Paris polyphylla*, and *Vanda bicolor* and prioritizing areas for possible future re-introduction. The present study was thus focused on the development of protocols for propagation and developing climate models for prediction of occurrence, re-introduction and prioritizing areas for conservation of the two threatened plant species of North East India.

Niche Modelling

During the present study MaxEnt protocol was used to develop the climate suitability model for distribution prediction as MaxEnt is a software specially designed to handle small sample size and presence only data. For *Paris polyphylla* two models were developed using two different sets of data and thus bringing out interesting insights on the climatic parameters like minimum and maximum temperature in post dehiscence period which plays a major role in the seed germination which are important determinants for the survival of the target plant. Model validation by ground truthing was also to bring out

new population not only in the study site but also in neighbouring states and countries bordering the target study area.

The model developed for *Vanda bicolor* showed the effectiveness of low sample size and climate data on MaxEnt model. Beside statistical significance of the model output, the model usability in real world application has been validated though ground truthing and testing of sites by introducing plants to predicted sites. The model besides pointing out new population also successfully located those existing population in neighbouring states and countries with success rate of 70% (calculated on stack developed using the MAXENT prediction map threshold value over the area of occurrence). Conservation related works are carried out for those species that are under high threat and those species in high threat category usually have low occurrence and it will be insignificant for conservationist unless working models are developed for these threatened species and the present study gives a good example of how low sample size can be used to develop effective prediction models. The present study on *Paris polyphylla and Vanda bicolor* strongly endorse the efficiency of MaxEnt models and their usage for species specific recovery plan and in identifying sites for in-situ conservation and re-introduction if the need arise.

Propagation

Propagation of *Paris polyphylla* was achieved by splitting of rhizomes with pre-existing lateral buds. Vegetative propagation by rhizome splitting is an age old practice. In the present study different types of *Paris polyphylla* rhizomes has been tested as planting material and excised rhizomes with lateral swellings or lateral bud primordia and well developed lateral buds were successfully able to regenerate into plantlets. The matured rhizomes used as planting material were also able to develop more lateral outgrowths which subsequently develop into lateral buds and branches forming clumps,

these lateral buds can be excised for further use as planting material. The impact of climate suitability on the growth and regeneration potential of *Paris polyphylla* was tested using the model developed. The response of plants to the different prediction threshold clearly confirms the need for a certain set of climate by plants to flower and produce seeds.

For *Vanda bicolor* micro-propagation was chosen as mode of propagation as contains millions of microscopic seeds without endosperm and micro-propagation techniques provides the seeds with all the necessary conditions for their germination, growth and development into plantlets. Immature seeds were used as explants for the initiation of culture on MS medium enriched with sucrose (0-3%, w/v), different level of plant growth regulators *viz.* α-naphthalene acetic acid (NAA) and N^6-benzyl adenine (BA) (0-9 µM) singly or in combination to obtain the optimum culture condition. Cultures initiated using immature seeds gives germination rate of 85% in a combined treatment of 3 µM NAA + 3 µM BA. Optimum PLBs initiation using *in vitro* raised shoots and roots was observed in 3 µM NAA + 3µM BA. Regeneration and development of plantlets was observed to be optimum in 3 µM NAA + 6 µM BA. In all the cases a combined treatment of both the PGR gives a better result than treated alone. Combination of 3 µM NAA + 3 µM BA, and in MS medium supplemented with 3% sucrose and activated charcoal (0.6% w/v) promoted healthier plantlets, and shoots having darker colour with excellent rooting. Hardened plantlets achieved survival percentage of 90% and the well established plantlets are transferred to natural habitat. The present study successfully exploits the potential of the vegetative ability of rhizome and tissue cultures develop a simple but efficient propagation for both the target species, the protocol thus establish will help in propagating the threatened target plant species.

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