Research Article

Prediction of high CPT yielding ecotypes of *Nothapodytes nimmoniana* (Graham) Mabb. in Western Ghats using Ecological Niche Modeling

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Abstract: Camptothecin (CPT) is one of anticancer drug that is widely used for treating various cancers. In India, the drug is primarily sourced from natural habitats of the red listed species *Nothapodytes nimmoniana*. Ecological niche models are potential tools to define and predict the “ecological niche” of a species that exhibit ecological variations. The predicted ecological niche of a species indicates their survival fitness against Bioclimatic variables. The habitat suitability was predicted using Maxent for different ecotypes of *Nothapodytes nimmoniana* (Graham.) Mabb. In this study the synonymised populations of *N. nimmoniana* in the Western Ghats were cogitated as five different ecotypes. The predicted habitat suitability of different ecotypes was evaluated and correlated against CPT content using high performance thin layer chromatography. The study shows a significant positive correlation between the predicted habitat quality and chemical content. The ecotypes growing in sites predicted as highly suitable showed high content of camptothecin compared to those growing in poorly suitable sites. Thereby enabling precise identification of “chemical hot-spots” which will eventually establish a strong foot hold on monoculture of the species, an effort towards conservation.

Key words: Ecological Niche Modeling, Ecotypes, Habitat Suitability Index, Camptothecin, High Performance Thin Layer Chromatography, *Mappia* complex, Western Ghats.

Introduction

Camptothecin (CPT) is the third most sought-after anticancer drug which was first discovered from the Chinese tree *Camptotheca acuminata*1–2. However, this drug was subsequently reported in India from Nothapodytes nimmoniana3. Cancer is not a rarity and is known to affect people all over the globe. According to world estimates the most commonly recorded cancers are lung cancer (12.7%), breast cancer (10.9%) and colorectal cancer (9.7%) of which breast cancer is the most prevalent cancer4–5. CPT is considered as one of the lifesaving drugs of the 21st century due to its wide range of anticancer activity6. Irinotecan (CPTII) and Topotecan (TPT), the two water-soluble semi-synthetic derivatives of CPT, have been approved by the United States Food and Drug Administration (FDA) for treating breast cancer, lung cancer, colorectal cancer and ovarian cancer7–11. They have also been approved by the FDA for the treatment of Acquired Immune Deficiency Syndrome12. *N. nimmoniana* (Graham.) Mabb. An indigenous Indian tree was formerly known as *N. foetida* (Wight) Sleumer and *Mappia foetida* Meirs. This small broad-leaved tree is predominantly distributed and endemic to the Western Ghats, though it has been reported from Sri Lanka, parts of Eastern (Assam) and Northern India (Himalayan foothills), Myanmar, China and Thailand12. In recent years especially in India, there has been an indiscriminate extraction of the trees from their natural populations due to the enormous demand for the chemical worldwide. This has led to a drastic population decline especially in the Western Ghats, earning it the “vulnerable status”14. The populations of *N. nimmoniana* in Western Ghats exhibit a wide array of morphological, genetical and physiochemical variations at the intra-specific levels12,15–16. These variations are a result of species complex in *N. nimmoniana* due to the merging of the five species of *Mappia* in India i.e. *Mappia foetida*, *M. oblonga*, *M. ovata*, *M. tomentosa* & *M. wightiana*3, under Asian genus *Nothapodytes* i.e. *N. nimmoniana*. In the present study these synonymized species were considered
as different ecotypes instead of either one species or synonymized species and the biomarker compound (CPT) was studied.

The niche of a species is a multidimensional ecological space controlled by the biotic and abiotic variables that are necessary for their survival, growth and reproduction\textsuperscript{18}. Ecological Niche Modeling (ENM) are predictive distribution models that combine the known geographic distribution of a species with environmental factors like climate and topography and thereby predict the possible spatial occurrence of a species\textsuperscript{19-20}. The overwhelming applications of ENM to diverse biodiversity processes such as predicting the habitat suitability and spatial distribution of a species\textsuperscript{21-23} has made it a well-founded and a reliable approach. Using these modeling techniques one can identify the areas that are most suitable to least suitable for the occurrence of the species\textsuperscript{24}. The plant species not only thrive well in the location predicted as good by ENM but also exhibit better fitness traits when compared to species in the poorly suitable habitats\textsuperscript{25-26}.

Secondary metabolites in plants are produced as a part of their normal physiological activity and or in response to abiotic or biotic stresses\textsuperscript{27}. They also confer fitness to the plants and serve as an adaptive trait during natural selection\textsuperscript{28}. Leaves, the major photosynthetic organs show vivid plastic responses in fluctuating growth conditions\textsuperscript{29}. In \textit{Nothapodytes nimmoniana} leaves serve as sites of CPT synthesis\textsuperscript{30} and also showed variation in their content according to geographical locations\textsuperscript{31}. Roja and Heble\textsuperscript{12} showed that the shoots of mature trees had 0.075% of CPT. Moreover, the presence of chiral ring in the CPT structure make it uneconomical for artificial synthesis of this compound at commercial level\textsuperscript{13}, which has paved way for the direct dependence of pharmaceutical companies for the compound from natural resources. Thus, in the present study we have developed a rational where ENM has been used as a probe tool to identify ecotypes that have high CPT content. These predictions will aid in unravelling biological hotspots of elite ecotypes with high CPT content, thereby strategizing conservation and commercial cultivation. The outcome of this study would serve as a direct platform in identifying suitable sites for agro economic purposes in turn reducing the pressure on the dwindling natural habitats of the species as a whole.

**Materials and Methods**

**Sample collection:**
The plant samples of the five ecotypes were collected from different parts of Western Ghats based on the literature. To map the distribution of the species primary data on the occurrence of the species was recorded during field visits using Global Positioning System (GPS) Garmin GLX – 12. These points were then digitized on to the GIS software i.e. Mapinfo Professional v.7.1 using the Southeast Asia layer from Mapmaker ver. 1.1.1.

**Chemical Extraction:**
Samples collected were oven dried at 65°C for 72 hours. Dried leaves (1g) were ground prior to extraction in 80% ethanol for 2 h at 50°C. Extraction was repeated with fresh 80% ethanol for a further 1 hr. The combined extracts were filtered through Whatman No. 1 filter paper and concentrated under reduced pressure at 20-40kPa at 45°C using a rotary evaporator (Buchi Rotovapor, Switzerland).

**HPTLC Analysis:**
Extracts were concentrated under vacuum and dissolved in methanol (made upto 1 ml) for HPTLC analysis. Standard (CPT) of different concentrations (Std C1-C5) were loaded along with samples on to the HPTLC plate (60 F\textsubscript{254}, E. Merck, Germany, 20.0 X 10.0 cm) with an automatic TLC applicator Linomat V with N\textsubscript{2} flow (CAMAG, Switzerland). The HPTLC plates were developed using ethyl acetate, Chloroform and methanol (5:4:5:0.5, v/v) as the mobile phase in the twin CAMAG trough glass tank. Each plate was developed to a height of 10 cm. After development, the plates were taken out and dried, then the spots were visualized in UV light (UV cabinet, CAMAG, Switzerland) at 254 nm and 366 nm respectively. CPT content was quantified using Camag TLC scanner 3 which is equipped with Wincat software. The plate was scanned at wavelengths of 366 nm UV (deuterium lamp) absorption-detection mode. A calibration curve was calculated by plotting concentration versus spot area of the compound. A known amount of plant
extract was spotted on TLC plate and CPT was quantified using the above calibration curve.

**Environmental data**

Nineteen Bioclimatic variables from the Worldclim database version 1.4 were used for developing Ecological Niche Models (www.worldclim.org). The data was downloaded from IPCC 3rd Assessment (www.ccafs-climate.org/data), at a spatial resolution of 1 km² (30 arc seconds). Bioclim values were extracted for each record and the co-linearity between the different Bioclim layers were assessed. From the ENM analysis a receiver operating characteristic (ROC) curve with mean and standard deviation for the various Bioclimatic variables was obtained.

**Ecological niche modeling:**

Using a global positioning system (GPS-Garmin) the latitude and longitude of 25 occurrence sites of *N. nimmoniana* in the Western Ghats were recorded. The sites were separated geographically ranging from 10 to 1500 km in the Western Ghats from Kanyakumari of Tamil Nadu state to Amboli Ghat of Maharashtra state. This data was used for modeling the species distribution.

Two ENM tools were used to develop the potential distribution and generating the habitat suitability for the species namely: a) Bioclim and Domain - based on presence only data and was run using the envelope method with the DIVA-GIS software b) Maxent, (Maximum entropy) – based on background points which minimizes the entropy. The softwares used were Maxent (version 3.3.2) and DIVA-GIS (version 7.1.7.2; http://www.divagis.org). The 19 Bioclimatic variables generated by Hijmans et al. were used (http://www.worldclim.org/) Both Bioclim and Maxent uses continuous probability scale and these measures were retained to derive the habitat suitability index (HSI) values. In the present study HIS was derived using the Maxent model only.

**Correlation of CPT content against Ecological niche predictions:**

To predict the potential niche map for *N. nimmoniana* the ENM tool, Maxent was employed. The programme was generally loaded with 19 BIOCLIM variables and utilizes existing Bioclimatic parameters from the occurrence sites of the species for prediction. After the Bioclimatic profile or parameters is obtained, the programme identifies all the possible locations on the grid with similar environmental characteristics to produce a map of the potential distribution of species. The niche map was developed at the resolution of 1 Km². The program generates the percentile values against the niche modeling information and predicts three categories of habitat suitability: Excellent, Medium and Low. To test the efficiency of the model prediction, the CPT content was estimated across three niche categories.

**Results**

**ENM predictions for *N. nimmoniana*:**

Development of spatial map for five ecotypes in Western Ghats:

Spatial map was generated using primary collection points from this study to show the distributional range of five different ecotypes (Figure 1). *M. foetida* was distributed throughout Western Ghats (Figure 1; circle points) and were found in the low altitudes. The map shows the distant distribution of *M. ovata* which was restricted to Northern Western Ghats (Figure 1; square points). Similarly, *M. wightiana* and *M. tomentosa* were also found within Nilgiri biosphere reserve (Figure 1; star and Triangle points respectively). *M. oblonga* was distributed sparsely with less population density in the hill slopes of Kerala and Karnataka (Figure 1; Diamond points).

**Figure 1.** Map shows the collection sites of different ecotypes of *Nothapodytes nimmoniana* from Western Ghats. Symbols are representing in the following such as square (*M. ovata*), circle (*M. foetida*), star (*M. tomentosa*), triangle (*M. wightiana*) & diamond (*M. oblonga*) respectively.

**Ecological niche modeling analysis:**

Niche modeling was performed using 120 points that were derived from literature, herbarium records and field surveys for *N.*
nimmoniana. Using the above points, the analysis was performed using both Bioclim and Domain algorithms of DIVA-GIS. However, the Domain algorithms of DIVA-GIS was not significant for the given presence data (Figure 2) as compared to Maxent (Maximum Entropy) analysis (Figure 3). The plots from each replication over bootstrap analysis were summarized into a grid file asc. file (Figure 3). The later file was imported into DIVA-GIS and the suitability index was derived as percentile values from 0-100 at the interval of 20 percentile. The predictions of habitat suitability were represented in five colours and then they were categorized into three; Red - Orange (High suitability), Yellow (Medium suitability) and light green - green (Low suitability). The habitat suitability index was derived for each ecotypes respectively based on the above percentile values.

**Figure 2.** Ecological Niche modeling predictions Bioclim and domain model in DIVAGIS. The percentile values for Habitat suitability index (HSI) were indicated in different colours for *Nothapodytes nimmoniana*.

**Figure 3.** Ecological Niche modeling predictions using Maximum Entropy model in Maxent. Habitat suitability Index (HSI) is represented in different colours.

**Calibration of models:**
The calibration test of the model for *N. nimmoniana* gave the AUC value of 0.993 ± 0.015 (Figure 4). Amongst the nineteen environmental variables, temperature seasonality (BIO4), isothermality (BIO3) and precipitation of the wettest month (BIO13) were the most influential contributing about 85% in Maxent model. Further, the rest of the sixteen layers of Bioclim together have contributed 15% to the habitat model of the species, of which mean temperature of wettest quarter (BIO8) and precipitation of wettest quarter (BIO16) collectively contributes 8.3% (Table 1). The temperature seasonality (BIO4) show the highest gain in the regularized training among the nineteen Bioclimatic variables in both the Jackknife as well as the AUC test.

**Table 1.** A heuristic estimate of relative contributions of the environmental variables for *Nothapodytes nimmoniana* in Maxent model

<table>
<thead>
<tr>
<th>Variable</th>
<th>Percent contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature seasonality (bio4)</td>
<td>49.3</td>
</tr>
<tr>
<td>Isothermality (bio3)</td>
<td>28.4</td>
</tr>
<tr>
<td>Precipitation of wettest month (bio13)</td>
<td>7.3</td>
</tr>
<tr>
<td>Mean temperature of wettest quarter (bio8)</td>
<td>6.2</td>
</tr>
<tr>
<td>Precipitation of wettest quarter (bio16)</td>
<td>2.1</td>
</tr>
<tr>
<td>Annual precipitation (bio12)</td>
<td>1.6</td>
</tr>
<tr>
<td>Mean monthly temperature (bio2)</td>
<td>1.4</td>
</tr>
<tr>
<td>Maximum temperature of coldest month (bio6)</td>
<td>0.8</td>
</tr>
<tr>
<td>Precipitation of warmest quarter (bio18)</td>
<td>0.7</td>
</tr>
<tr>
<td>Precipitation of driest quarter (bio17)</td>
<td>0.7</td>
</tr>
<tr>
<td>Precipitation of coldest quarter (bio19)</td>
<td>0.4</td>
</tr>
<tr>
<td>Precipitation seasonality (bio15)</td>
<td>0.3</td>
</tr>
<tr>
<td>Maximum temperature of warmest month (bio5)</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Chemical profiling of CPT using HPTLC:
The presence of camptothecin (CPT) in the plant extract was detected on HPTLC plate at Rf value of 0.49 ± 0.01 in methanol mobile phase which was validated by comparing with Rf of the standard pure camptothecin procured from Sigma Aldrich (ab120636) (Figure 5). The chromatograms of camptothecin from plant samples were obtained and compared with standard chromatogram of pure camptothecin on the basis of retention factor and peak area (Figure 6).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean temperature of driest quarter (bio9)</td>
<td>0.2</td>
</tr>
<tr>
<td>Temperature annual range (bio7)</td>
<td>0.2</td>
</tr>
<tr>
<td>Mean temperature of coldest quarter (bio11)</td>
<td>0.1</td>
</tr>
<tr>
<td>Annual mean temperature (bio1)</td>
<td>0</td>
</tr>
<tr>
<td>Precipitation of driest month (bio14)</td>
<td>0</td>
</tr>
<tr>
<td>Mean temperature of warmest quarter (bio10)</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 4. The graph shows the significance of AUC (Area Under Curve) between test and train data for *N. nimmoniana*.

The 25 individuals from five ecotypes that were analysed for active ingredient camptothecin showed significant variations in camptothecin content ranging from 0.673 to 9.697% (w/w) (Figure 7; Table 2). The results showed that significantly high mean CPT content was recorded in the ecotype of *M. wightiana* (6.38% SD ±3.15) followed by the ecotype *M. tomentosa* with 4.296% (w/w) (Figure 7). The above two ecotypes are restricted to Nilgiri biosphere reserve of Western Ghats (Figure 1). But, when all these 25 points were plotted against the derived HSI values, the correlation between the CPT content and habitat suitability was significant at p<0.05 (P-value= 0.009526; R²= 0.052; df= 24; Figure 8).

Figure 6. HPTLC profile of Camptothecin based on overlaid chromatograms using 3D densitogram to show its presence at 366nm.

Figure 5: TLC Plate shows the lanes of samples loaded along with five different concentrations (C1–C5) of camptothecin to identify and quantify the compound in multi-individuals of five ecotypes of *N. nimmoniana*.

Figure 7. Chemical profiling of Camptothecin using HPTLC analysis across different ecotypes of *Nothapodytes nimmoniana*.
Figure 8. Correlation of chemical yield (CPT) against habitat suitability index (HSI) based on HPTLC analysis and ecological niche modeling. The P-value is 0.009526 and significant at p<0.05.

Discussion

Ecological niche predictions:

Several studies have validated the fact that habitat predicted as a highly suitable niche for any given species fosters enhanced growth and survival fitness characters. The present study is also in congruence with the above findings in that the ecotypes of both *M. wightiana* and *M. tomentosa* from Nilgiri biosphere reserve show high content of CPT when compared to ecotypes (*M. foetida*) growing in low suitable habitats. Therefore, it can be concluded that the secondary metabolites (CPT) are synthesized as a by-product of normal growth and metabolism of the plants rather than a response to either physical stress or biotic stress.

Comparison of niche models:

Several studies have evaluated or tested the efficiency of the model prediction by comparing them with respect to their regularization and training the presence data. For instance, Peterson et al. compared two algorithms Maxent and Bioclim and showed striking difference between them, and finally concluded that, BIOCLIM predicts with increased commission error whereas Maxent reconstructs the overall distribution of the species at the lowest threshold. However, in this study, we employed two ENM tools namely DIVA-GIS and Maxent to develop the niche map using 120 primary points. There was a difference between the niche maps developed by these two algorithms and eventually the Maxent prediction was used for the interpretation of chemical data from HPTLC analysis which was mainly due to the significant AUC values against the Bioclimatic variables compared to other models.

Using niche model for the prospecting of secondary metabolites:

The major goal of the present study was to explore the possibilities of using ENM for prospecting of ecotypes that produce high levels of secondary metabolites. Hence, ENM was done using the known occurrence (120) points based on primary and secondary data. The predictive distribution map developed using maximum entropy method for Western Ghats adopting Maxent agreed with the observed distributional pattern of the species. Only high rainfall hilly slopes of the Western Ghats, Nilgiri biosphere reserve were predicted to be highly suitable for the species. This indicates the high habitat-specificity and the restricted distribution of certain ecotypes i.e. *M. wightiana, M. tomentosa* that were corroborated from this study. The excellent niche for the occurrence of *M. wightiana, M. tomentosa* (high percentile with red colour pixels in the map) has indeed laid the platform for prospecting sources of CPT in the future. This attempt of considering the five synonymised species of *N. nimmoniana* as different ecotypes to assess the chemical content (CPT) is first of its kind, though studies at population levels have been carried out.

Defining eco-parameters for high CPT content:

The application of ENM has raised the importance of understanding and estimating the factors that determine the geographic distribution of species. However, in this study, we estimated the eco-parameters against the secondary metabolite (CPT) with the background of 19 Bioclimatic variables. The result thus indicates that following variables (eco-parameters) i.e. Temperature seasonality (bio4), Isothermality (bio3), Precipitation of wettest month (bio13), Mean temperature of wettest quarter (bio8) and Precipitation of wettest quarter (bio16) are highly influential in the production of CPT. This study throws light on not only the ecotypes that have high CPT content but has also predicted suitable habitats where cultivation of these ecotypes could be carried without destroying the natural habitats.

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References


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