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GENETIC DIVERSITY OF NEPENTHES GRACILIS IN HEATH FOREST IN SOUTH AND CENTRAL PART OF KALIMANTAN

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Abstract

Nepenthes gracilis* found the lowlands in the form of peat swamp forest, heath forest and widely distributed in Thailand, Sumatra, Peninsular Malaysia, Singapore, Kalimantan and Sulawesi. Unique characteristics of the plant, potential benefits and threats of declining population of *N.gracilis* impacted this plant as a conservation priority in the heath forest. Characterization of *N.gracilis* applied before implementing conservation actions. The conservation approach proceeded by analyzing the genetic diversity of *N.gracilis* from various types of heath forest. The research object used as genetic material is the leaf part of *N.gracilis*. The sampling locations of *N.gracilis* were in the heath forest of Guntung Ujung village, Gambut sub-district, Banjar regency, South Kalimantan (Location 1), heath forest in Tabalong Regency, South Kalimantan (Location 2), heath forest in East Kotawaringin Regency, Central Kalimantan (location 3), heath forest of the Nyaru Menteng Arboretum, Palangkaraya, Central Kalimantan (location 4). The tools and materials used for genetic analysis with PCR-RAPD markers are divided into several stages of work, namely: DNA extraction, DNA quality and quantity testing, DNA visualization and data analysis. The results show that OPP-09 and OPBH-20 were the primers with the highest amplification success for *N.gracilis* plants from the heath forest and the amplification values are 52.5% and 51.25%. The percentage of polymorphism bands were 97.62% and 95.24%, respectively. Disturbed heath forest with open canopy cover showed an indication of low genetic diversity shown by *N.gracilis* originating from location 1 and location 4 of heath forests and the highest genetic diversity value and the highest % locus polymorphism value (79%) was shown by *N.gracilis* location 3 with relatively undisturbed forest cover and classified as old growth. It is crucial to preserve the heath forest cover from damage in order to maintain high genetic diversity of *N.gracilis*. Genetic relationships between populations can be a barometer in conservation programs for *N.gracilis*.

Keywords: *Nepenthes gracilis*, heath forest, genetic diversity, DNA extraction, DNA quality, DNA visualization

The authors may not translate the abstract and keywords into Chinese themselves.

I. INTRODUCTION

Pitcher plant is a type of understorey and a carnivorous plant that thrives by absorbing nutrients [1], also known as insectivorous species. Nepenthes is a species that characterizes heath forests [2]. The existence of Nepenthes is an indicator of the low nutrients contained in the soil. The most common type of pitcher plant found from various types of heath forest is Nepenthes gracilis (*N.gracilis*). *N. gracilis* is also commonly known in Borneo [3],[4] especially related to soil type, light and water content [5]. The density of *N.gracilis* in the heath forest varies depending on the character of its growing habitat [6]. Nepenthes is the largest genus of pitcher plant species with a distribution ranging from northern Australia throughout South-east Asia to Southern China. Nepenthes can be found in tropical lowland evergreen rain forest, heath forest, peat swamp forest, montane forest and limestone forest [7]. [8] reported that there are 100 species of Nepenthes, with the widest distribution in the islands of Indonesia, Philippines, Borneo and Sumatra.

The natural distribution of *N.gracilis* includes Thailand, Sumatra, Peninsular Malaysia, Singapore, Kalimantan and Sulawesi. This species is often associated with shrubs *Gleichenia linearis* and *Baeckia sp*. *N.gracilis* is found in the lowlands in the form of peat swamp forest, heath forest which is still good and open, open swamp to an altitude below 100 m and can reach 800 m above sea level. *N.gracilis* is rarely found at altitudes > 1000 m. This plant is included in the terrestrial climber with a height of up to 5 m. The stems are triangular in diameter from 1.5-5 mm and the length between the stem segments is 2.5-9 cm. The leaves are thin with a length of between 10-25 cm and a width of 2 cm. The pouch is green, red or green with red spots. The flowers can be white, green, pink or brown [9].

Nepenthes plants on land with low N, P, K content [10]. As a mechanism to overcome the limited availability of nutrients in the soil, insect predation is a strategy to meet the nutrient needs of pitcher plants. Nutrients, apart from being obtained from the soil, are also absorbed from the process of predation of insects trapped in their pockets. This specific phenomenon plays an important role in nutrient acquisition [8].

Residents of South Kalimantan as food wrappers use *n.gracilis*. The stem is used as a rope, the liquid in a closed bag is used as eye drops, ulcer and asthma medicine and the boiled water of the roots is used as a diabetes medicine [11]. The pitcher plant in Kalimantan used by the community as ornamental plants, ropes, traditional medicines, cake containers made of rice sticks, drinking water sources, and flower bouquets [12].

The liquid in a sealed bag used by the local people of Khasi and Garos (India) as drops for red eyes, itchy eyes, to cure cataracts and night blindness. The liquid also used to treat stomach ailments, diabetes and gynecological diseases. The sealed bag and its contents made in the form of a paste are used for various skin ailments, including leprosy/leprosy and sometimes mixed with rice beer to reduce urinary problems and blockages. The open bag and its contents are also made in the form of a paste and mixed with water to be given to cholera sufferers [13]. Semar bags have been used in traditional medicine to treat various diseases, such as dysentery, abdominal pain, swollen eyes, and bedwetting in children [14].

Bioactive compounds from Nepenthes can function as antifungal, anti-bacterial and other activities [15]. Nepenthes has activity as an antibacterial, antifungal, antimalarial and anti-diabetic [16]. Nepenthes has the ability to absorb heavy metals [17].

The increasing forest destruction makes Nepenthes habitats being disturbed and most of pitcher plant species may become easily lost [18]. Nepenthes populations in the wild are predicted to decline due to factors such as forest fires, illegal logging, conversion of forest land or shrubs into residential areas, shifting cultivation, plantation, agriculture, or mining and forest reclamation for oil palm plantations [19].

Discovering plant diversity, structure, and composition of vegetation in forest ecosystems is essential to understand the mechanism of species coexistence and forest dynamics in a unique and vulnerable ecosystem such as tropical heath forest [20]. Specific characteristics of the plant, potential benefits and threats of declining population of *N.gracilis* make this plant a conservation priority in the heath forest. Characterization of *N.gracilis* needs to be done before implementing

conservation actions. The conservation approach can be preceded by analyzing the genetic diversity of *N.gracilis* from various types of heath forest.

II. METHODS

The research object used as genetic material is the leaf part of *N.gracilis*. The sampling locations of *N.gracilis* were in the heath forest of Guntung Ujung village, Gambut sub-district, Banjar regency, South Kalimantan (Location 1), heath forest in Tabalong Regency, South Kalimantan (Location 2), heath forest in East Kotawaringin Regency, Central Kalimantan (location 3), heath forest of the Nyaru Menteng Arboretum, Palangkaraya, Central Kalimantan (location 4). Laboratory analysis was carried out at the Genetic Laboratory of the Department of Silviculture, Faculty of Forestry, IPB, Laboratory of Geospatial Information Systems, Faculty of Forestry, University of Gastric Mangkurat for making a map of the research location.

The protected forests of Guntung Ujung Village (Location 1) and East Kotawaringin Regency (location 3) represent degraded forest conditions in the form of covering open bush areas. Kerangas Forest in Tabalong District represents secondary heath forest (Site 2). The heath forest of the Nyaru Menteng Arboretum, Palangkaraya, represents the old growth heath forest (Location 4). The map of the research location is shown in Figure 1.

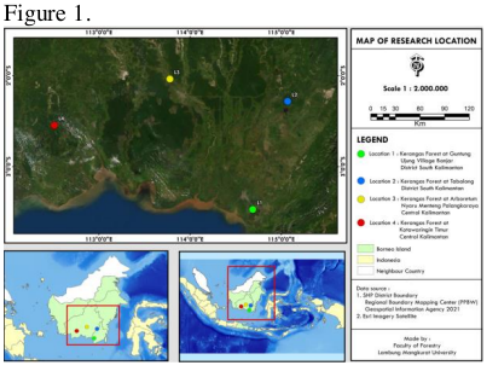

Figure 1. Location of Sampling of *N.gracilis*

Each location made several observation points with the number of observation points is 4 points (total observation points are 16 points). Placement of observation points is done purposively. Each observation point took 5 leaf samples from different individuals with the distance between sampling points ranging from 5-10 meters, so the number of samples taken was 80 individuals. An overview of the individual semar bag sampling technique for genetic analysis is shown in Figure 2.

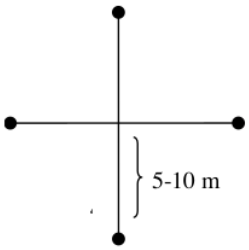

Figure 2. Sampling of individual semar bags for genetic analysis

The tools and materials used for genetic analysis with PCR-RAPD markers are divided into several stages of work, namely: DNA extraction, DNA quality and quantity testing, DNA visualization and data analysis. Table 1 presents some of the equipment and materials used in PCR-RAPD. DNA analysis on semar bag leaves was carried out using the random amplified polymorphic DNA (RAPD) method. The RAPD method consists of two stages, namely DNA extraction and RAPD analysis.

Table 1. Materials and tools of the PCR-RAPD technique

Analisys	Stages			
	Extraction	**PCR**	**DNA Visualization**	**Data analysis**
RAPD	**Tools:** rubber gloves, scissors, 1.5 ml tube, permanent marker, mortar, pestel, micropipette, tips, tube rack, vortex, centrifugation machine, water bath, freezer, desiccator. **Ingredients:** extract buffer, 2% PVP, chloroform IAA, cold isopropanol, NaCl, 95% ethanol, TE buffer.	**Tools:** 0.2 ml tube, permanent marker, stationery, micro pipette, tips, centrifugation machine, PTC-100 PCR machine. **Ingredients:** DNA, aquabidest, H2O, RAPD primers, Taq polymerase.	**Tools:** micropipette, tips, centrifugation machine, electrophoresis bath, agar mold, erlenmeyer, gloves, UV transillumination, DNA photo equipment. **Ingredients:** *agarose,* 1x TAE buffer, 1x TBE, 10x blue juice, DNA marker, EtBr	**Tools:** computer, POPGENE software version 1.31, and Microsoft excel

DNA extraction was carried out using the Cetyl Trimethyl Ammonium Bromide/CTAB method [21]. Leaf samples at the base of 1 cm x 1 cm, the middle 2 x (1 cm x 1 cm) and the ends of 1 cm x 1 cm were crushed in a clean pestel. The results of the scour were then transferred to a 2 mL tube, then 500-700 l of extract buffer solution was added (a mixture of Tris-HCl, EDTA, NaCl, CTAB, and water) and 100 l of 2% PVP. Furthermore, the incubation process carried out in a water bath for 45-60 minutes at a temperature of 65oC and then cooled for ± 15 minutes. Added 500 l of chloroform and 10 l of phenol, then the mixture centrifuged to make it homogeneous at 10,000 rpm for five minutes. The aqueous phase is taken using a micro pipettes and transferred to a new tube, this water phase is used, while the organic phase is stored in the freezer. Furthermore, 500 l of cold isopropanol and 300 l of NaCl were added, then stored in the freezer for 45-60 minutes to obtain DNA pellets. The next activity was DNA washing process by adding 300 l of 100% ethanol, then centrifuged at 10,000 rpm for five minutes. The DNA pellet was stored in a desiccator for ± 15 minutes and after that, 20 microliters TE buffer solution was added, vortexed and then centrifuged again. Furthermore, visualization of DNA by electrophoresis method. The agarose gel used is 1% with TAE buffer solution. Mix 3 l DNA pellet and 2 l blue juice. Running electrophoresis was carried out by inserting a mixture of DNA and blue juice using a micro pipettes into the agarose gel well. After the running process, followed by staining using Ethidium Bromide (EtBr) (A mixture of 10 l EtBr and 190 ml distilled water) for 15 minutes. Then the photo shoot was done using a UV transilluminator detection.

The RAPD PCR reaction was carried out using 15 l of a solution volume consisting of 2.5 l of H20 (nucleus free water), 1.5 l of each primer, 7.5 l of Go Taq Green Master Mix Kit (Promega), and 2 l. DNA template. DNA amplification was carried out using a PTC-100 Progammable Thermal Cycler (MJ Research, Massachussetts, USA). The RAPD process carried out using five primers. The temperature settings on the PTC-100 machine for the RAPD reaction are: i) pre-denaturing at 950C for 3 minutes, ii) denaturing at 950C for 1 minute, iii) annealing at 350C for 2 minutes and 720C for 2 minutes iv) extension at 720C for 10 minutes. This process carried out or repeated for 35 cycles. The PCR results were then electrophoresed with 2% agarose gel in 1 x TAE buffer solution and stained with Ethidium Bromide (EtBr).

The results obtained photographed and analyzed by scoring the banding pattern that appeared. The results of the photo interpretation then analyzed using the POPGENE 32 software Version 1.31. Genetic variability was estimated using the following parameters: percentage of polymorphic loci; number of observed alleles (na); number of effective alleles (ne); expected heterozygosity (He), genetic differentiation (Gst).

III. Results and Discussion

A total of 5 primers were used in the RAPD analysis. The resulting descriptions of the RAPD markers used to estimate the genetic diversity of *N.gracilis* in the heath forest are shown in Table 2a and table 2b..

Table 2a. Description of the various RAPD markers used

Primary names	Base arrangements	Monomorphic band	Polymorphic band	Total Amplification
OPP-09	5'-GTGGTCCGCA-3'	1	41	42
OPP-15	5'-GGAAGCCAAC-3'	0	24	24
OPP-19	5'-GGGAAGGACA-3'	5	24	29
OPBH-16	5'-CTGCGGGTTC-3'	0	17	17
OPBH-20	5'-CACCGACATC-3'	1	40	41

Table 2b. Description of the various RAPD markers used

Primary names	Base arrangements	% of polymorphic band	total samples	Amplification success (%)
OPP-09	5'-GTGGTCCGCA-3'	97.62	80	52.5
OPP-15	5'-GGAAGCCAAC-3'	100	80	30
OPP-19	5'-GGGAAGGACA-3'	82.76	80	36.25
OPBH-16	5'-CTGCGGGTTC-3'	100	80	21.25
OPBH-20	5'-CACCGACATC-3'	95.24	80	51.25

OPP-09 and OPBH-20 were the primers with the highest amplification success values of 52.5% and 51.25%, while the percentage of polymorphism bands were 97.62% and 95.24%, respectively. An overview of the characterization of RAPD markers can be seen in Figure 3.

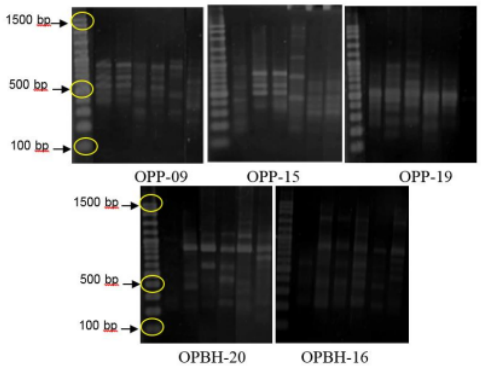

Figure 3. DNA bands of the RAPD marker on *N.gracilis*

The base pair range for the five primers is from 150 bp – 1400 bp. The size of this base pair is within the range of results obtained by the study using the five primers using the RAPD technique, which is in the range of 150 – 2800 bp to characterize the genetic diversity of *N.khasiana* [13].]. The base pair range for several primers in the genetic diversity analysis of Nepenthes spp. that using RAPD technique ranged from 150 bp to 1.450 bp [22]. Relatively few monomorphic bands were found in the RAPD analysis using these five primers. The percentage of polymorphic band is 82.76%. The DNA band at the top and far from the well is a DNA group with a light molecular weight, so the number of base pairs is relatively low. RAPD is known as a genetic marker technique that can produce a large number of DNA band polymorphisms. Several genetic studies using RAPD markers have found large polymorphism bands produced [23], [13], [24], [25].

Based on the results of data processing, a description of the genetic diversity of *N.gracilis* in the heath forest is listed in Table 3.

Table 3. Results of the analysis of population genetic diversity of *N.gracilis*

Location	N	polymorphic locus	% polymorphic locus	na	ne	He
Diversity in-population						
Guntung Ujung (Location 1)	11	31.5	63%	16.29	14.87	0.26
Tabalong (Location 2)	9	35.5	70%	16.99	14.92	0.29
Nyaru Menteng (Location 3)	14	41.5	79%	17.90	15.41	0.31
Kotawaringin Timur (Location 4)	9	26	56%	15.59	14.22	0.23
Diversity inter-population (Gst) = 0.27 (N=43, lokus polimorfik= 106, % lokus polimorfik= 99.3%)						

Information:
N = Number of samples; Na= Number of alleles observed; Ne= The number of effective alleles; He= Genetic diversity

The results listed in Table 3 show that the overall diversity of *N.gracilis* between populations is relatively high with a Gst value > 0.15 [26]. The value of diversity in the population will increase if the results of the analysis use fewer primary data. An increasing trend also occurs in the value of % polymorphism loci. The highest genetic diversity value was shown by *N.gracilis* in the Nyaru Menteng heath forest (Location 3). The % value of polymorphism loci was also the highest for *N.gracilis* also located in the Nyaru Menteng heath forest (79%). The second highest genetic diversity of *N.gracilis* came from the Tabalong shellfish forest (Location 2), the value of % polymorphism locus = 70%. The relatively high genetic diversity at the Nyaru Menteng location is related to the relatively low disturbance of old growth forest cover. The genetic diversity of *N.gracilis* was high at location 2, where the vegetation cover was secondary heath forest. *N.gracilis* found in the heath forest of Guntung Ujung Village (Location 1) and East Kotawaringin (Location 4) had the lowest genetic diversity. Both types of heath forest are open heath with a high level of disturbance.

Degradation of forests leads to reduced genetic variation and hampers the flow of genetics. The fragmentation of the forest causes high kinship and potential for inbreeding. Repeated fires caused the population of *N.gracilis* to decrease in both locations. Damage or pressure on the habitat will reduce the genetic diversity of *N.gracilis*, because only *N.gracilis* with certain genetic characters will survive with increasingly extreme heath habitats. Loss of genetic variation results in disturbances in the frequency of genetic distribution which will hinder the potential for evolution in the adaptation process to environmental changes [27].

The description of genetic relationship between populations was then analyzed further using the Ntsys 2.0 program. The data used is the result of Popgene's analysis using 5 primers. A description of the genetic relationship relationships between genetic populations is shown in Figure 4.

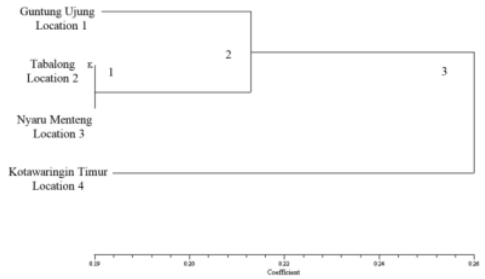

Figure 4 Diagram of *N.gracilis* genetic relationship in the heath forest.

N.gracilis found in Location 2 and Location 3 have the closest genetic relationship with a genetic distance of 9.32% and the coefficient of identity difference between the two is small (0.19). The low level of disturbance has resulted in the persistence of large genetic variation in these two heath forest populations. The closest relationship between *N.gracilis* from heath forest Location 1 is with *N.gracilis* from location 2. The genetic distance formed is 22.15% (10.83+1.51+9.32).

This finding is supported by the history of heath forest formation between Location 1 and Location 2 which is associated with the Barito river terrace. The genetic distance that is farthest from other locations is location 4. The existence of natural barriers such as rivers is thought to limit the genetic distribution of a species. The biggest river barrier that occurs is between the village heath forest Location 1 and Location 4. There are 5 major rivers that separate these two locations, namely the Barito river, Kapuas river, Kahayan river, Katingan river and Mentaya river.

RAPD primers OPP-9 and OPBH-20 were primers with the highest polymorphic loci compared to the other three primers. Based on the results of the genetic relationship analysis using 5 primers, the genetic relationship description shown is indicated in Figure 4. Meanwhile, the results from the other three types of primers show different genetic relationship descriptions. These results conclude that these two primers play a role in the description of the *N.gracilis* relationship in the heath forest. Further analysis was carried out by selecting the primers used for genetic relationship analysis (using 2 primers OPP-9 and OPBH-20). Based on the % of polymorphic loci formed and the similarity of the pattern of genetic relationship formed, the OPBH-20 primer was the best primer in revealing the *N.gracilis* genetic relationship in the heath forest. Primer with GC 60% is the best primer for RAPD analysis (Bhau et al. 2009). OPP-9 and OPBH-20 primers were primers with GC percentage 60%.

The results of this genetic diversity analysis show that in general the genetic diversity of *N.gracilis* is still relatively high. Knowledge of population structure is very important for the conservation of natural populations while maintaining the total evolutionary potential and minimizing genetic relationship [28]. Understanding genetic diversity is essential for establishing effective and efficient conservation and breeding practices.

The role of humans is to exchange genes between populations or genetic inputs that are far apart can be done to conserve the genetic diversity of *N.gracilis*. Conserving *N.gracilis* at each location in the heath forest which is now in a fragmented forest condition is very important. In situ conservation can be done to enrich the genetic diversity of *N.gracilis* in the heath forest in Guntung Ujung Village. Based on the genetic distance approach and genetic diversity possessed, the use of *N.gracilis* plants originating from the old growth heath forest of Nyaru Menteng, Palangkaraya and the secondary heath forest of Tabalong as a source of genetic material can be done. The findings in this study will also be taken into consideration for forest rehabilitation, because genetic diversity will be maintained with relatively undisturbed forest stands as a whole.

IV. Conclusion

The RAPD markers can be used to estimate the genetic diversity of *N.gracilis* in the heath forest. OPP-09 and OPBH-20 were the primers with the highest amplification success in the RAPD genetic marker method for *N.gracilis* plants from the heath forest. The amplification values are 52.5% and 51.25%. The percentage of polymorphism bands were 97.62% and 95.24%, respectively. The highest genetic diversity value and the highest % locus polymorphism value (79%) was shown by *N.gracilis* located in the Nyaru Menteng Palangkaraya heath forest location, Central Kalimantan (location 3) with relatively undisturbed forest cover and classified as old growth. The existence of natural barriers such as rivers is limitting the genetic distribution of a species. Disturbed heath forest with open canopy cover showed an indication of low genetic diversity shown by *N.gracilis* originating from location 1 and location 4 heath forests. It is important to maintain forest cover from damage in order to maintain high genetic diversity of *N.gracilis*. Genetic relationships between populations can be an indicator in conservation programs for *N.gracilis*. Conserving *N.gracilis* at each location in the heath forest in a fragmented forest condition is very important. Based on the genetic distance approach and genetic diversity possessed, the use of *N.gracilis* plants originating from the old growth heath forest as a source of genetic material can be done. The results of this study can be taken into consideration for forest rehabilitation since genetic diversity will be maintained with relatively undisturbed forest stands as a whole.

Acknowledgment

The guidelines for citing electronic information as offered here are in modified illustration of the adaptation by the International Standards Organization (ISO) documentation system and the American Psychological Association style (APA, https://apastyle.apa.org/).

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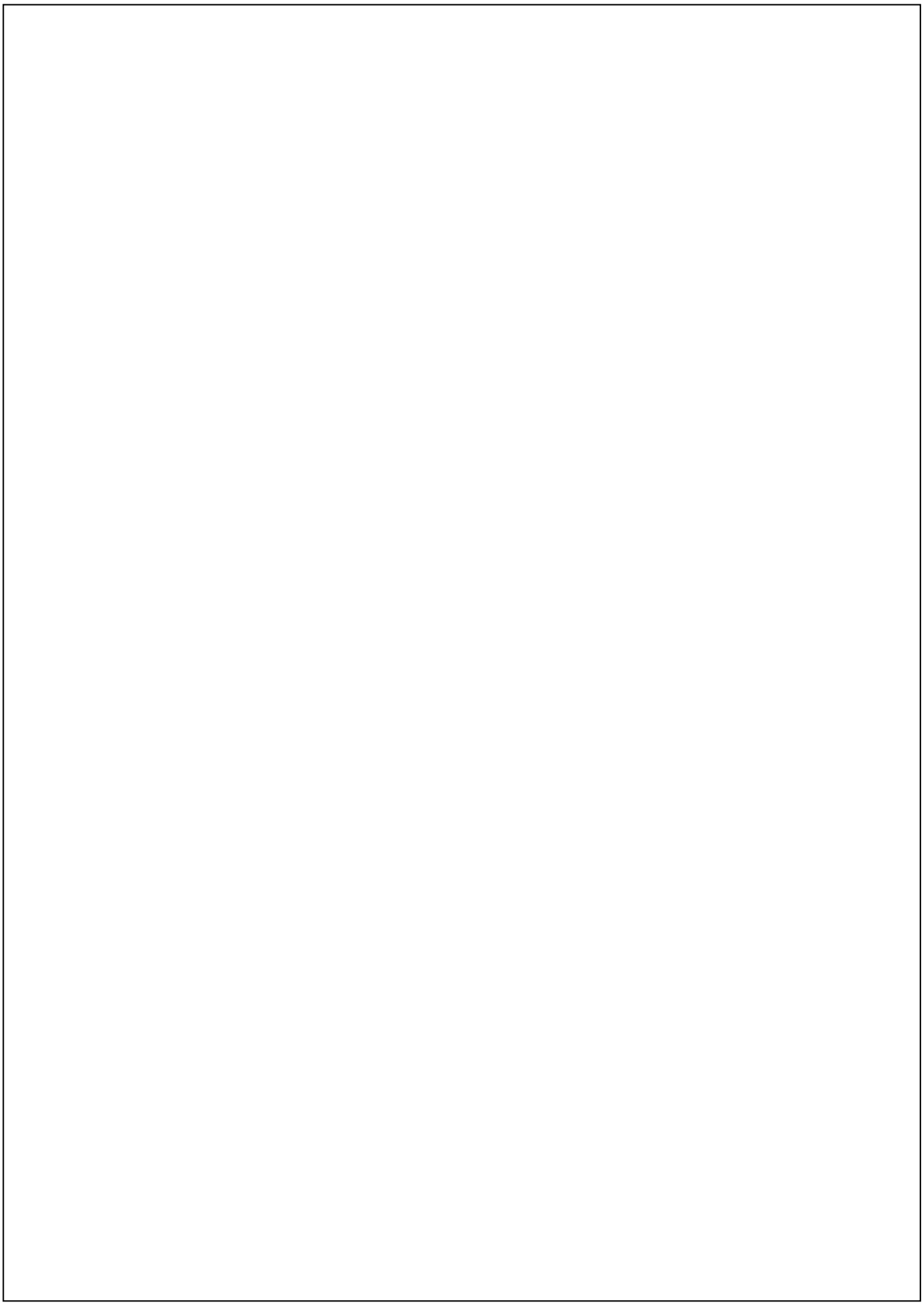

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