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Characterization of *MatK* and *RuBisCO* Genes within the Population of *Chrysophyllum albidum* (G. Don) in Akure Forest Reserve, Ondo State, Nigeria

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ABSTRACT
Genetic diversity study of tree species is important because it gives species a better chance of survival in the face of climate change. This study investigated the genetic diversity within the population of *Chrysophyllum albidum* in Queen's plot, Akure forest reserve, Ondo State, Nigeria using *MatK* and *RuBisCO* genes. Trees of *Chrysophyllum albidum* were identified, counted, their coordinates and diameters at breast height were recorded. Foliar samples from each tree were collected, cleaned and stored in a sealed nylon containing silica gel as dehydrating agent. Genomic DNA was extracted using Cetyltrimethyl ammonium bromide (CTAB). Polymerase Chain Reaction (PCR) was performed in a volume of 25µl in a DNA thermal cycler. PCR products were evaluated using Brilliant Dye™ Terminator Cycle Sequencing Kit and sequence data were analyzed using BIOEDIT and MEGA X software. The number of *Chrysophyllum albidum* (32 trees/ha) found in Queen's plot was two times more than what was recorded in the previous studies. *Chrysophyllum albidum* trees were found in all the diameter classes. This indicates that this species has good regeneration status. The phylogram, constructed using *RuBisCO* and *MatK* genes for the sampled *Chrysophyllum albidum* trees, revealed the intra-specific variation of this species in Queen's plot. The information provided in this study could be used to breed *Chrysophyllum albidum* with superior characteristics. Therefore, serious conservation efforts should be implemented and sustained to avert any loss of the identified genetic diversity.

Keywords: *Chrysophyllum albidu;* Conservation; *MatK* Gene; Phylogram; and *RuBisCO* Gene

INTRODUCTION
Molecular markers have since been used to understand an evolutionary trends at the genus level (Barbara et al., 2021) and are currently been used for genetic diversity study of tree species in south west Nigeria (Boboye et al., 2021; Fasalejo et al., 2021; Lawal et al., 2023). Restriction fragment length polymorphism (RFLP), Random amplified polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP), Inter simple sequence repeat (ISSR), Sequence characterized amplified region (SCAR), Simple sequences repeat (SSR), Single nucleotide polymorphism (SNP), are common molecular markers that could be used for genetic variation studies. However, combination of *ribulose-1,5-bisphosphate carboxylase/oxygenase* (*rbcL*) and maturase K (*matK*) genes was recommended as a barcode standard for plants (CBOL, 2009). This is because they efficiently recover quality sequences and provide a high level of plant species discrimination (Burgess *et al.*, 2011).

Matk gene, which is important for early chloroplast development and seedling survival (Lv et al. (2020), has a very high rate of nucleotide substitution and provides high phylogenetic signal for resolving evolutionary relationships and relatedness among plants at all taxonomic levels (Hilu et al., 2003). Asahina et al., (2010) revealed that rbcL gene has a low level of mutation when compared with other barcodes in cpDNA. The low level of mutation is the superiority of the *rbcL* gene and as such could be used for an in-depth study of intraspecies genetic and phylogenetic variations. Generally, trees are sources of timber for construction purposes (Ramage et al., 2017). Some tree species have medicinal and timber values as well as the production of edible fruits for sustainable livelihood. *Chrysophyllum albidum*, also referred to as "African Star Apple," is one of the few tree species in this category (Amusa et al., 2003; Orwa et al., 2009; Houessou, 2012), timber and edible fruit production (Onyekwelu et al., 2011).This fruit tree grows as a wild plant and belongs to the family Sapotaceae. Ecologically, the tree has efficient nutrient cycling and the high rate of mineralization of the leaves improves the quality of the topsoil (Adesina, 2005). The fruit pulp is widely consumed and thus plays a significant role in food security (Onyekwelu and Stimm, 2011). According to Onyekwelu et al., (2011), *C. albidum* fruit serves as a delicacy and another source of food, income, and rural employment through their collection and sale. Other parts of *C. albidum* (such as leaves, seeds, roots, and bark) are also used for curing different diseases such as diabetes, ulcer, sterility and sexual weakness, blood pressure and asthma, etc. (Houessou, 2012).

Deforestation and forest degradation has decimated many important tree species in south West Nigeria (Lawal and Adekunle, 2013). Asides, the domestication of this important fruit tree (Fig. 1) is still its low ebb as farmers currently consider planting crops that will provide income almost immediately. Of the two strict nature reserves in South West Nigeria, *C. albidum* was only found in Queen's plot, Akure forest reserve (Lawal and Adekunle, 2013). In a study of the floristic composition and diversity in Omo Biosphere Reserve, no species of *C albidum* was recorded (Salami and Akinyele, 2018; Ubaekwe, 2020). Similarly, this important fruit tree was not found in some prominent sacred grooves in South West Nigeria (Onyekwelu et al., 2022).

Obviously, this fruit tree species is threatened with extinction in this region owing to deforestation and conversion of forested land to agricultural farmland, hence the need to conserve the remnant trees. From an evolutionary perspective, the extent of genetic variation among individuals can set a species' adaptive potential and influence its persistence over time. This study therefore was deigned to investigate the genetic diversity of *C. albidum* population in Queen's plot, Akure forest reserve, Ondo State, Nigeria using *MatK* and *RuBisCO* genes.

Figure 1. *Chrysophyllum albidum* (A = Tree; B = Leaves; C = Fruits; D = Seeds)

MATERIALS AND METHOD

Study Area

Akure Forest Reserve is a protected area situated between Aponmu and Obada communities in Akure South Local Government of Ondo State, Nigeria, covering 66 km^2, at latitude 7.296 °N and longitudes 5.03 °E. The Akure forest reserve has a tropical climate with prominent wet/rainy and dry seasons: The rainy season generally occurs between March and October while the dry season occurs between November and February yearly. Also, the mean annual temperature is about 26 °C (minimum 19 °C and maximum 34 °C) (Adekunle et al., 2013).

Method of data collection

A systematic line transect was used for data collection (figure 2). Three line transects and eight plots were sampled in Queen's plot. A square plot of 50 m by 50 m was laid in an alternate position along each transect. A total of twelve plots were used. In each plot, the diameter at the breast height (DBH) measured at 1.3 m above the ground of each matured *C. albidum*s pecies was recorded. The coordinate of each tree was recorded with a global positioning system (GPS) and their leaves were sampled, cleaned and stored in a sealed nylon containing silica gel. The dried leaf samples were taken to the Biosafety Postgraduate Research Laboratory of the Federal University of Technology Akure for DNA extraction, and Inqaba Biotech West Africa Ltd, Ibadan, Oyo State, Nigeria for subsequent analysis.

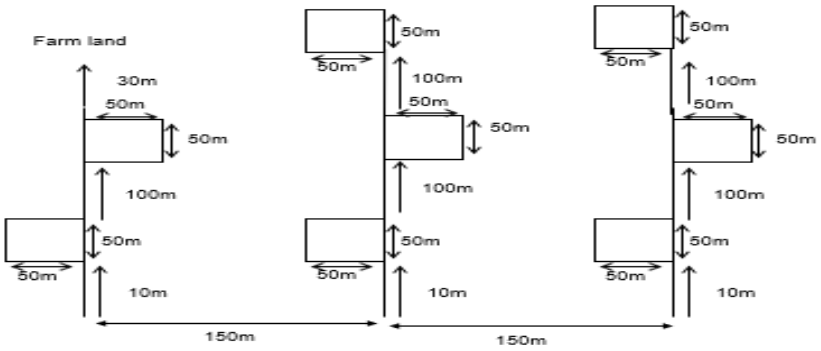

Figure 2: Systematic line transect used in Queen's plot data collection

DNA extraction

DNA extraction processes were carried out in the Biosafety Postgraduate Laboratory of the Federal University of Technology Akure. To carry out this practical, Doyle and Doyle's (1990) DNA extraction protocol were adopted as described below.

Fifty milligram (50 mg) samples of young leaf tissues were grounded to a fine powder with a mortar and pestle.The powdered sample was placed in 2-mL microtubes containing 700 µL 2 % CTAB extraction buffer. The solution was incubated at 65 ºC for 45 minutes, gently mixing by inversion every 15 minutes; 500 µL of chloroform-isoamylalcohol (24:1) was added to the tube and gently mixed for 1 minute. Sample was centrifuged for 10 minutes at 12,000 rpm. A 500µL of the supernatant was transferred to a fresh tube with 700 µL of cold isopropanol (-20^{0}C), gently mixed by inversion and centrifuged at 12,000 rpm for 10 minutes. The liquid solution was released and the DNA pellet was washed with 700 µL of 70 % ethanol to eliminate salt residues adhered to the DNA and set to dry for 12 h, with the tubes inverted over a filter paper, at room temperature. Thereafter, the pellet was re-suspended in 100 µL TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) plus 5µL ribonuclease (RNase 10 mg µL^{-1}) in the tube.This solution was incubated at 37 ^{0}C for 1h, and stored at -20 ^{0}C before subsequent analysis.

Polymerase chain reaction

DNA was amplified by PCR using RuBisCO (rbcL) (F: 5′-TGAAAACGTGAATTCC CAACCGTTTATGCG-3′; R: 5′-GCAGCAGCTA GTTCCGGGCTCCA-3′)and MatK (MATK 390F: CGATCTATTCATTCAATATTTC; MATK 1326R: T CTAGCACACGAAAGTCGAAGT).PCR reactions were performed in a volume of 12.5 µl in a DNA thermal cycler (Eppendorf, mastercycler gradient). The reaction mixture contained is as presented in Table 1.The amplification conditions are as presented in Table 2.

PCR products were cleaned using an enzymatic method (ExoSAP). The ExoSAP master mix was prepared by adding 50 µl of 20U/µl Exonuclease I (Catalogue No. NEB M0293L) and 200 µl of 1U/µl of Shrimp Alkaline Phosphatase (Catalogue No. NEB M0371)to a 0.6ml micro-centrifuge tube. The reaction mixture was mixedand incubated at 37 ℃ for 15mins and at 80℃ for 15mins. A 10 µl amplified PCR Productwas mixed with 2.5 µl ExoSAP Mix and sequenced using the Nimagen, Brilliant Dye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions. The labeled products were cleaned with the ZR-96 DNA Sequencing Clean-up Kit. The cleaned products were injected into the Applied Biosystems ABI 3500XL Genetic Analyzer with a 50 cm array.

Data analysis

Chrisophylum albidum trees were classified into diameters classes and their spatial distribution was done using Quantum GIS (QGIS) while the sequence data were analyzed using BIOEDIT Version 7.2.6. (Informer Technologies Inc, 6800 Altamor Drive Los Angeles, CA 90045 United States) and MEGA X software (Tamura et al., 2021).

RESULT AND DISCUSSION

A total of 32 trees per hectare of *Chrysophyllum albidum* were found in the study area. These trees were unevenly distributed across the diameter classes. The highest number of *Chrysophyllum albidum* trees were found in 10-15 cm and 26-30 cm diameter classes as presented in Figure 3. Four trees of this species were found in the highest diameter class. Out of the 32 trees per hectare uncovered in the study area, four trees were found in 16-20cm, 21-25cm and 31.35cm diameter classes as shown in Figure 3.

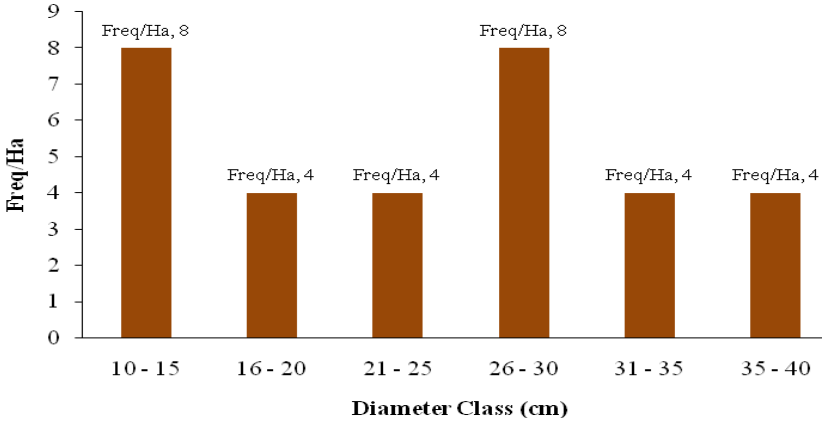

Figure 3: Diameter distribution of *Chrisophylum albidum* in the study area

Figure 4 indicates the geographical map of the sampled area showing the Global Positioning System (GPS) locations of each *Chrysophyllum albidum* species sampled while Figure 3 shows the diameter distribution chart for all species identified.

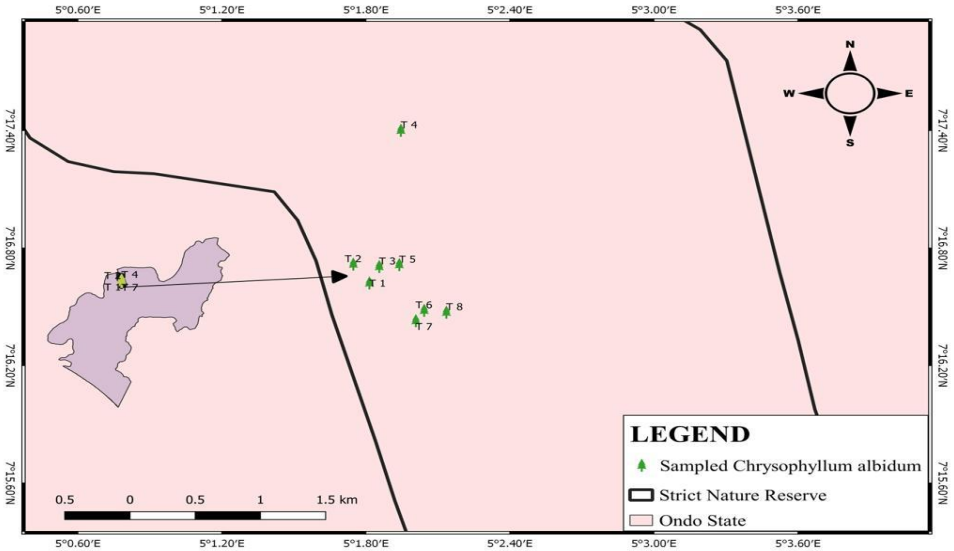

Figure 4: *Sampled* Chrysophyllum albidum *in Queen's plot, Akure forest reserve*

The phylogram for the evolutionary relationship among the sampled *Chrysophyllum albidum* in Queen's plot, Akure forest reserve, is presented in Figure 5. Based on *RuBisCO* gene, *Chrysophyllum albidum* in the study area were distinctly classified into two phylo-groups and three subgroups. The phylogenetic tree reveals no speciation event among trees 1, 6 and 7. These trees have common ancestral lineage, they were genetically similar and as such were put together as a clad. Similarly, no speciation event among trees 3, 4 and 5. Tree 2 was the ancestral parent while trees 3, 4 and 5 were the descendant of that ancestor. Considering the horizontal branch for trees 1, 6 and 7, there was no variation in their genetic make-up. Similarly, trees 3, 4 and 5 had no variation in their genetic make-up.

The phylogram for the sampled *Chrysophyllum albidum*in Queen's plot is presented in Figure 6. Based on *MatK* gene, sampled *Chrysophyllum albidum* in Queen's plot were put into two phylo-groups with only "*C. albidum* Tree 6" in a group. All the sampled trees originated from one ancestral parent, *C. albidum* Tree 6. *C. albidum* Tree 1 and *C. albidum* Tree 2 were more genetically similar than any other sampled trees. *C. albidum* Tree 5 was the ancestral parent for *C. albidum* Tree 1, *C. albidum* Tree 2, *C. albidum* Tree 3 and *C. albidum* Tree 4 as shown in Figure 6. Similarly, *C. albidum* Tree 1, *C. albidum* Tree 2 and *C. albidum* Tree 3 originated from *C. albidum* Tree 4.

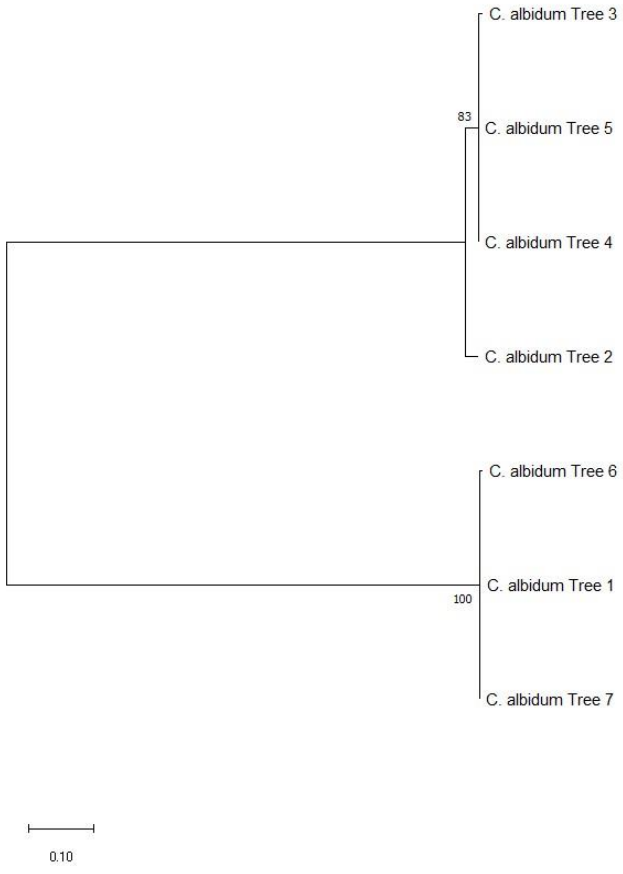

Figure 5: Phylogenetic relationship among *Chrysophylum albidum* in Queen's plot, Akure Forest Reserve using *RuBisCO* Gene

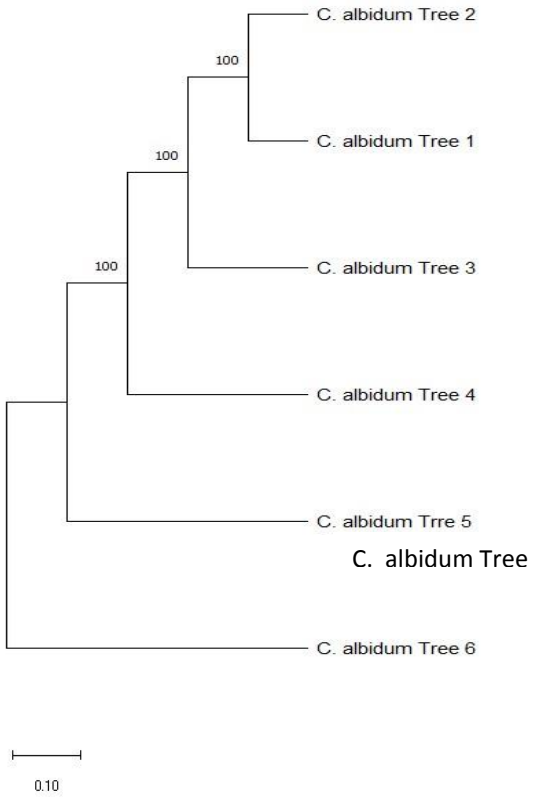

Figure 6: Phylogenetic relationship among *Chrysophylum albidum* in Queen's plot, Akure Forest Reserve using MatK Gene

DISCUSSION

Genetic variation as an integral part of biological diversity needs special attention and monitoring to ensure effective conservation while safeguarding the sustainability of forest ecosystems (Fussi et al., 2016). This can only be possible through unravelling the genetic variation within and among the population of tree species in a forest ecosystem (Lawal et al., 2023). Akure forest reserve contains several tree species (Lawal and Adekunle, 2013; Lawal et al., 2021) and serve as a source of various goods and services for livelihood sustenance in Ondo state (Adekugbe et al., 2020). Unfortunately, our forested lands have been declining progressively owing to the various activities of man in the bid for economic development (FRA, 2010). Forest degradation, fragmentation, and conversion of the forests to other forms of land use in Nigeria are currently advancing at worrisome rates, especially in the Akure forest reserve. Adedutan and Olusola (2015) reported that in the last two decades, a significant portion of the undisturbed forest and Forest Reserves in the 80s had lost.

Thirty-two (32) trees of *Chrysopyllum albidum* per hectare were discovered. The number of *Chrysopyllum albidum* found in Queen's plot in this study was more than two times what was previously recorded (12) in 2013 (Lawal and Adekunle, 2013). This result reveals that the population of this species in Queen's plot has been increasing. An increase in the frequency of a particular species could mean an increase in genetic diversity, which is a fundamental requirement for evolution and adaptation (Hague and Routman, 2016).

However, this species, until now, has been enlisted among the endangered tree species in Nigeria (Formecu, 1999). Morover, Onyekwelu and Stimm (2011) opined that *Chrysophyllum albidum* has a high probability of going into extinction in the nearest future and this could affect the livelihood of millions rural dwellers that depend on them as a substitute for food, medicinal uses and a source of household income.

This study also revealed that *Chrysopyllum albidum* trees were found in all the diameter classes. More so, the highest number of trees was in the lowest diameter class (10-15 cm) and (26-30 cm) class respectively. This is an indication that this species has good regeneration status. Onyekwelu *et al.* (2022) opined that, the knowledge of plant regeneration status helps in developing management options and setting priorities and that a limited tree regeneration is a major threat to forest sustainability. Khum-bongmayum et al. (2006) revealed that, the satisfactory natural regeneration behaviour of the forests depends on population structure characterized by the production and germination of seeds, establishment of seedlings and saplings in the forest. The diameter distribution of this important fruit tree also indicated that the forest will continue to supply the fruits and other services derivable from the tree of *Chrysopyllum albidum* particularly if the current level of protection is sustained.

The phylogram, constructed using *RuBisCO* and *MatK* genes for the sampled *Chrysopyllum albidum* trees, further revealed the intra-specific variation of this species in Queen's plot. The genetic variation recorded in this study could be attributed to the conservation efforts of the Forestry Research Institute of Nigeria (FRIN) and Ondo State Government. The findings of this study disagree with the work of Boboye et al. (2018). They revealed that the genetic base of *Chrysopyllum albidum* population in Osun State has been seriously eroded and recommended an urgent step by individual, organizations and government to conserve the genetic base of this important tree species. Osun State has savanna climate. Therefore, tree species found in Osun State may have to survive harsher environmental conditions as a result of climate change than those found in rainforest ecosystem, where this study was carried out. Drastic environmental changes could lead to the loss of adaptability of some trees in this species and a reduction in their genetic diversity is expected. Also, *Chrysopyllum albidum* population in Queen's Plot, Akure forest reserve is under the strict protection of Ondo State government and the federal government of Nigeria.

CONCLUSION

A total of 32 trees per hectare of *Chrysopylluma lbidum* were found in Queen's plot. They were distributed across all the diameter classes. *Ribulose-1,5-bisphosphate carboxylase/oxygenase* (*RuBisCO*) gene classified the sampled trees into major phylogenetic groups. Similarly, *Megakaryocyte-Associated Tyrosine Kinase* (*MatK*) gene further revealed the diversity among the sampled trees. This study provides valuable information on the current status of *Chrysopyllum albidum*. This information, if properly harnessed, could be used to breed *Chrysopyllum albidum* with superior characteristics such as early fruitability, sweet and big sized fruits. Conservation efforts therefore should be sustained to avert any loss of the identified genetic diversity.

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Supplementary Materials

The supplementary materials are available online at www.bestjournal.com

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary materials

Table 1. Polymerase Chain Reaction Amplification Protocol

Component	**Volumes for a 12.5µL reaction**
Template DNA	2.00 µL
10µM Forward Primer	0.25µL
10µM Reverse Primer	0.25µL
One Taq Quick Load 2X Master Mix with Standard Buffer	6.25µL
Nuclease free water	3.75 µL

Table 2. Thermal Cycling Protocol

Step	**Stage of PCR**	**Temperature (°C)**	**Time (min: secs)**
1	Initial Denaturation	94	5:00
2	Denaturation	94	0:30
3	Annealing	52	1:00
4	Extension	68	1:30
5	Final Extension	68	10:00
6	Hold	4	hold