24th - 25th May 2010

Biotechnology: How far have we gone?

Organized by:
Department of Molecular Biology
Faculty of Resource Science and Technology

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Opening Ceremony Speech by Assoc. Prof. Dr. Edmund Sim Ui Hang

Associate Professor Dr. Edmund Sim Ui Hang
Advisor, The 3rd Biotechnology Colloquium (Biotech Col3)
Department of Molecular Biology

There are basically two modes in the evolution of any university department. The easier mode is one that is totally focused on the academic processes of undergraduate development, leaving research and postgraduate activities as side efforts. The arduous and trickier mode is none other than an emphasis (individual and concerted) on research and publication. Naturally, the latter mode of evolution is the hallmark of a true university department, and one that has a bona fide scholarly profile. On this premise and the evidence of a successful 3rd Biotechnology Colloquium, the Department of Molecular Biology has truly become a genuine scholarly institution. Three years running and three times successful in bringing out the best in research from our researchers and postgraduate students. Congratulations and well done to all our research teams and the coordinators of this year's colloquium.

A special tribute of recognition is extended all the postgraduate students of the Department of Molecular Biology – without which this series of yearly colloquiums (and proceedings thereof) would not have been possible. Most departments take pride in the successful graduation of their postgraduates. For this department, we are beyond words in praise and honour for our students who do not just fulfil the role of studentship but that of scholarship. In essence, the participation and presentation in colloquiums have become inducted as part of the research lifestyle among our postgraduate students. The culmination of these into the compilation of another Colloquium Proceedings is not just evident of a vibrant research culture but also the proper fulfilment of our postgraduate programmes. At the Department of Molecular Biology, we dare to presume that employability of our successful postgraduates cannot be based on their thesis alone, but on the fact that they have gained competency in all the main sectors of research. These include presentation in research seminars and the involvement in publication.

On a final and lasting note, if it is right for an individual researcher is to be acknowledged for good performance, it is even more right for a department to be recognised and rewarded for excellence in their corporately-achieved research accomplishments. Until such award exists in our organisation, we should savour the glory (in thoughts) that the Department of Molecular Biology can be the recipient for three years in a row.

Well done, to the Department of Molecular Biology!

Keynote Address by Professor Dr. Kasing Apun

Invited Keynote Speaker

Professor Dr. Kasing Apun
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ECO-ZOONOSES PROJECT IN SARAWAK: HUNTING FOR ENTERIC BACTERIA IN WILDLIFE

Kasing Apun[1], Lesley Maurice Bilung[1], Mohd. Tajuddin Abdullah[2], Kho Kai Ling[1], Chen Yik Ming[1], Adom Benjaman[1], Mustafa Abdul Rahman[2] and Samuel Lihan[1]
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ABSTRACT
Wildlife is known to be major hosts to pathogenic microorganisms that are important to human health. A wide variety of domestic and wild animal species can act as reservoirs for these pathogens, which may be viruses, bacteria, fungi or parasites. Changes in the natural environmental conditions has been reported to bring about changes to the population structure, distribution and population dynamics of wildlife that can enhance the spread of potentially zoonotic disease in a local community, leading to the emergence of infectious disease. The key event in the emergence of most emerging infectious diseases (EIDs) is a change in host-parasite ecology. This research was therefore aimed at examining the relationships between ecosystem disturbance, occurrence and diversity of zoonotic pathogens in selected free-ranging wildlife species found herein. This multidisciplinary zoonoses study was confined to potentially infectious agents found in selected small mammals and birds. The bacterial zoonoses group focuses on a study of the bacterial enteric pathogens including enteropathogenic *E. coli*, *Salmonella*, *Campylobacter* and *Shigella* as free-ranging wildlife species such as rodents and migratory birds are often symptomatic carriers of the enteric bacteria that have the potential to transmit disease. Although the risks and threats of infectious agents are largely acknowledged by the general public, there is an obvious lack of necessary data and information for improved understanding of the nature, implications and precautionary requisites of EID, particularly for Borneo Island. This paper will discuss recent findings of our eco-zoonoses project in Sarawak that examine the presence (or otherwise) of these potential infectious pathogens in the wildlife across different landscapes, both disturbed and intact habitats along the Rajang River basin, the largest river basin in the state of Sarawak (and in Malaysia) that has been subjected to extensive land use changes. Subsequently, the knowledge from this study will be used to further improve the surveillance and response strategies to detect, prevent and mitigate the impact of these pathogens on local human health.

PLENARY 1:

ISOAMYLASE DEBRANCHING ENZYMES FOR STARCH METABOLISM

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ABSTRACT

Isoamylases are debranching enzymes that hydrolyze -1,6 linkages in -1,4/-1,6–linked glucan polymers. In plants, they have been shown to be required for the normal synthesis of amylopectin, although the precise manner in which they influence starch synthesis is still not fully understood. Three distinct isoamylase isoforms (Stisa1, Stisa2, and Stisa3) have been identified. The expression patterns of the genes are consistent with the possibility that they all play roles in starch synthesis. Analysis of the predicted sequences of the proteins suggested that only Stisa1 and Stisa3 are likely to have hydrolytic activity and that there probably are differences in substrate specificity between these two isoforms. Partial purification of isoamylase activity from potato tubers showed that Stisa1 and Stisa2 are associated as a multimeric enzyme but that Stisa3 is not associated with this enzyme complex. The data suggest that Stisa1 and Stisa2 act together to debranch soluble glucan during starch synthesis. The catalytic specificity of Stisa3 is distinct from that of the multimeric enzyme, indicating that it may play a different role in starch metabolism such as breakdown of starch granules.

PLENARY 2:

FROM CONSERVATION TO INNOVATION: BUILDING RESEARCH CAPACITY FOR PLANTED FOREST DEVELOPMENT IN SARAWAK

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ABSTRACT

The increase in global demand for wood requires increase in forest productivity. The alternative is to farm trees in plantations composed of fast-growing species with short rotation cycle (6-8 years). The rationale is that natural forests at the most produce about $3m^3$/ha/yr of commercial timber, whereas plantations can produce annually from $10m^3$/ha of hardwoods to $30m^3$/ha of softwoods and thus, decrease the effects of human pressure on our ecosystems while increasing the competitiveness of Sarawak's forest industry. This is in line with State Government's aspiration to establish one million hectares of planted forests by year 2020 to meet the increasing demand from both domestic and international markets for raw materials. It is estimated at least 30 million seedlings are required for annual planting or reforestation programmes. In this regard, the forest genomics research will help respond to the need to develop adequate tools that enable us to produce quality planting materials that are of faster growth, high-yield and high wood quality, and also adapted to local conditions, so that we may achieve economic benefits of great significance. Realizing the needs, we have centered our research on the development of tools via biotechnological innovations for tree breeders. We have successfully developed: 1) an array of highly informative and polymorphic DNA markers specific for identifying the genetic makeup of two fast growing indigenous tree species, i.e. Kelampayan and Sawih; 2) the one step 'Touch-incubate-PCR' approach for preparing plant tissues for high throughput genotyping, and 3) a genomic resource database, aka CADAMOMICS (10,368 ESTs) for wood formation in Kelampayan via high-throughput DNA sequencing. These tools will greatly facilitate the selection of quality planting materials for planted forest development in Sarawak as well as long-term tree improvement activities by integrating genomics into our breeding programme via association mapping. The overall benefit of genomics application to tree improvement programme will be in terms of greater certainty in the outcome of results, specifically the performance of the forest plantations, as well as the savings in time and cost in the production and supply of quality planting materials.

Key Words: forest biotechnology, genomics, plantation forestry, tree improvement, *Neolamarckia cadamba*, *Duabanga moluccana*, association mapping

INTORDUCTION

Rapid socio-economic changes in the world are having profound impacts on all sectors, including forestry. While wood products demand is increasing, so is the demand for environmental services of forests. The increasing demand is triggered by population growth and rise in income (gross domestic income). Global demand for wood products is projected to increase from 3.5 billion m^3 in 1990 to 6.4 billion m^3 in 2020. Apart from that, the demand for environmental services of forest is also increasing whereby more natural forests will be excluded from wood production, and recently the bioenergy policy, the use of biomass, including wood is increasingly encouraged (Figure 1). Natural forests are unable to meet current global demand for wood due to the long generation intervals and slow growth rate, resulting in the loss and degradation of natural forests by logging (Fenning and Gershenzon, 2002). About 12 million ha of forests are lost every year. Africa, South East Asia and South America provide the best environment for tree growth but account for more than75% of total losses.

Therefore, the increased demand for wood is likely to require increased forest productivity. The alternative is to farm trees in plantations composed of fast-growing species with short rotation cycle (6-8 years). With more research, particularly in the production of improved planting materials, tree selection and improvement, the plantations could produce more of the industrial timber by the end of the next 2 decades. The rationale is that natural forests at the most produce about 3 m^3/ha/yr of commercial timber, whereas plantations can produce annually from 10 m^3/ha of hardwoods to 30 m^3/ha of softwoods. Furthermore, plantations are easier to manage due to the mono or double species mix compared to very diverse natural forest stands (Krishnapillay and Razak, 2001). Hence, plantations development will serve as a strategy for maintaining a sustainable supply of timber and at the same time, reducing the logging pressure on natural forests for wood production to an acceptable level.

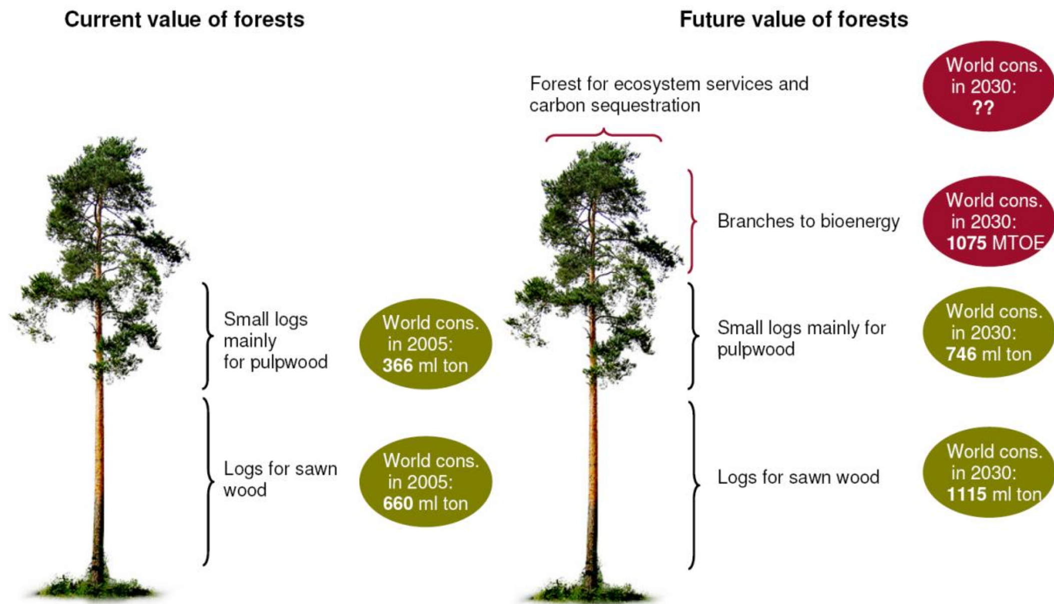


Figure 1: The current and future values of forests (Source: FAO, State of World Forests 2009)

In Sarawak, the state government has introduced the Forest (Planted Forest) Rules (1997) to encourage the development of commercial forest plantations and has set a target of 1.0 million hectares for forest plantations to be established by 2020. It is estimated that 30 million of high quality seedlings are required for the annual planting programme. Realizing the needs, a joint research programme (URL: http://fgilab.com) focusing on two selected fast growing indigenous tree species, namely *Neolamarckia cadamba* (Kelampayan) and *Duabanga moluccana* (Sawih) has been carried out to provide solutions, in addressing the shortage of quality planting materials for licensed planted forest areas in the state. The programme covers R&D activities on forest genomics, biotechnology and tree improvement geared towards enhancing commercial plantation forests as well as sustainable management of forest resources in Sarawak (Ho, 2008). Furthermore, the programme via the use of state-of-the-art technologies and approaches will help respond to the need to develop adequate tools for producing trees that are better adapted to local conditions, so that we may achieve economic benefits of great significance. In this paper, we would like to highlight the projects that have been conducted with special focused on molecular genetic studies. Among others are the development of highly informative and polymorphic DNA markers specific for identifying the genetic makeup of two fast growing indigenous tree species, i.e. Kelampayan and Sawih; 2) the one step 'Touch-incubate-PCR' approach for preparing plant tissues for high throughput genotyping, and 3) a genomic resource database, aka CADAMOMICS (10,368 ESTs) for wood formation in Kelampayan via high-throughput DNA sequencing.

Why *Neolamarkia cadamba* and *Duabanga moluccana*?

Neolamarkia cadamba (Roxb.) Bosser, locally known as Kelampayan belongs to the family of Rubiaceae, has been identified as a promising fast growing species for planted forest development in Sarawak. Kelampayan is a large, deciduous and fast growing tree species, thus with characteristics which guarantee early economic returns within 8 to 10 years. Under normal conditions, it reaches a height of 17 m and diameter of 25 cm at breast height (dbh) within 9 years. It is a lightweight hardwood with a density of 290-560 kg/m^3 at 15% moisture content. Thus, kelampayan is one of the best sources of raw material for the plywood industry, besides pulp and paper production (Joker 2000). It can also be used as a shade tree for dipterocarp line planting, whilst its leaves and bark have medical application. The dried bark can be used to relieve fever and as a tonic, whereas a leaf extract can serve as a mouth wash (Mondal *et al*., 2009; Patel and Kumar, 2010). Another plantation tree species, *Duabanga moluccana* Blume or Sawih from the family of Sonneratiaceae can grow up to 45 meters tall and 100 cm in diameter. This tree is of great economic importance for the production of various wood works and products such as plywood, veneer and pulping. Additionally, it is also suitable for interior paneling, matches, moulding and pulping (CIRAD Forestry Department, 2003).

'Touch-incubate-PCR' Approach (aka *f*asTip-X) for High Throughput Genotyping

The *f*asTiP-X approach is a rapid extraction method which allows direct amplification without going through conventional CTAB extraction. In the same time, it allows DNA extraction without contacting any harmful chemicals and liquid nitrogen. This method offers a great advantage whereby it requires only approximately 20 minutes for DNA preparation before PCR amplification thus increases the possibility for high-throughput genotyping. Apart from that, the requirement of small amount of plant material is greatly suitable for samples with limited quantity.

We have tested the *f*asTiP-X approach by using the 5S rRNA primers via PCR on 4 different species, namely *Neolamarckia cadamba* (Roxb.) Bosser (Kelampayan), *Duabanga moluccana* (Sawih), *Durio zibelthinus* (Durian) and *Dimocarpus longan* Lour. (Longan). The amplification of DNA template obtained from the *f*asTiP-X was comparable to the positive control which extracted using conventional CTAB method (Figure 2). The PCR analysis using DNA template isolated by the *f*asTiP-X approach for each species was repeated 3 times to prove the reliability and reproducibility of this method. This result shows that *f*asTiP-X approach was more suitable for high-throughput genotyping compared to the conventional DNA extraction considering its rapidity, simplicity and cost-effective features (Table 1).

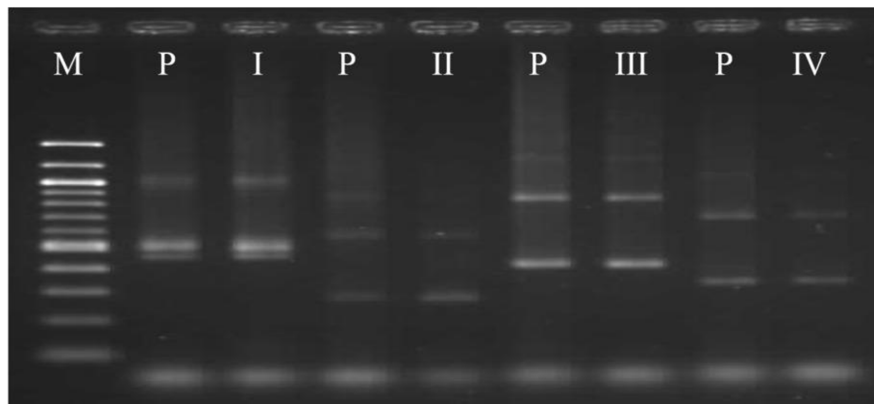


Figure 2: PCR amplification using 5S rRNA primers with template obtained from *f*asTiP-X. M: 100bp marker; P: positive control; I: *Neolamarckia cadamba*; II: *Duabanga moluccana*; III: *Durio zibelthinus*; and IV: *Dimocarpus longan* Lour

Table 1: Comparison between conventional CTAB method and *f*asTiP-X approach in the preparation of samples for high-throughput genotyping

Item	Conventional CTAB method	*f*asTiP-X approach
Size of leaf sample	Large	Small
Condition of leaves	Fresh / Dried	Fresh
Time (before PCR)	~ 2 days	~ 20 minutes
Cost (before PCR)	RM 25-30	RM 3-5
Long-term storage	-20°C	-20°C
DNA Quantification	Yes	No

Development and Polymorphism of Simple Sequence Repeat (SSR) markers

The development of an ideal molecular DNA marker system which is genetically co-dominant and multi-allelic is becoming a major concern due to the genetic complexity of breeder's populations and high levels of heterozygosity in individual genotypes (Elizabeth *et al*., 2003). As such simple sequence repeat (SSR) marker is the ideal marker system of choice for the tree breeders. For instance, in our previous study four SSR loci were successfully used to determine genetic relatedness among selected mother trees of *Shorea leprosula* and *Dipterocarpus cornutus* (Wickneswari and Ho, 2003). We found that four and three selected mother trees of *S. leprosula* and *D. cornutus*, respectively were not closely related and therefore, could be used as

potential seed sources for an advanced breeding programme and tree plantations. However, the availability of SSR markers especially for indigenous tree species is still limited due to the high development cost.

In the present study, we used ISSR-Suppression PCR method in developing the SSR markers for Kelampayan and Sawih (Figure 3). In total, 31 SSR primer pairs were designed to flank the targeted SSR in the Kelampayan genome. Among the 31 SSR loci, 7 (22.6%) SSRs were classified as the perfect type, 11 (35.5%) as the imperfect type and 13 (41.9%) with the compound type. The most abundant dinucleotide motif found in the Kelampayan genome was TG/AC and CT/GA repeats. Meanwhile in Sawih, 44 SSR regions have been identified, i.e. 20 SSR regions (48%) were identified from Lian *et al*. (2001) and another 24 SSR regions (52%) were based on Lian *et al*. (2006). The most numerous class was the perfect compound with 24 (54.5%) occurrences, followed by the imperfect compounds with 8 (18.2%), simple perfect with 8 (18.2%), and the simple imperfect repeats with 4 (9.1%). Majority of the dinucleotide microsatellites found in the Sawih genome was CT/GA repeats (83.3%) followed by CA/GT repeats (16.7%) (Table 2). These newly designed SSR markers will facilitate molecular breeding programme for the improvement of both species by providing useful baseline genetic information for effective selection of plus trees, provenance trials, and establishment of forest seed production areas (SPAs) in the selected forest reserves.

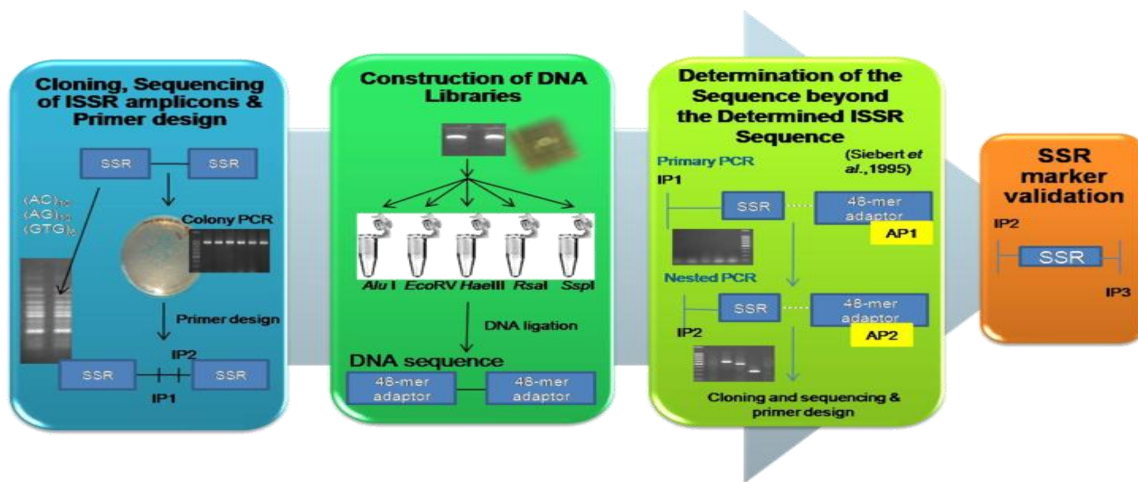


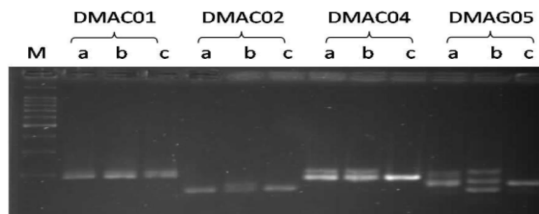
Figure 3: ISSR-Suppression PCR method in developing SSR markers for Kelampayan and Sawih

Table 2: Some of the SSR loci and its repeat motif for Kelampayan (NC-) and Sawih (DM-)

Locus	Repeat motif*
NC-AG01	$(CT)_n$
NC-AG03	$(GT)_n$
NC-GTG01	$(TG)_n(CG)_m$
NC-GTG10	$(GCT)_n C(CTT)_m$
NC-GTG04	$(TCA)_n$
NC-AC05	$(TGG)_n TTA(TGG)_m$
NC-AC11	$(CT)_n (CCCT)_m$
NC-AC14	$(TG)_n AA(TG)_m$

* n and m show the repeated nucleotides

Locus	Repeat motif*
DM-AC01	$(GA)_nCACC(GA)_m$
DM-AC02	$(GT)_nGC(GT)_m$
DM-AC04	$(AT)_n(GT)_m$
DM-AG05	$(CT)_n$



Transcriptomics and Bioinformatics on Wood Formation of Kelampayan

Despite the high economic value of tropical wood, little is known about the genetic control of wood formation or xylogenesis for Kelampayan compared to loblolly pine (59,797 ESTs), poplars (25,218 ESTs) and spruce (16,430 ESTs) (Whetten *et al*. 2001). Wood or secondary xylem is manufactured through the process of cell division, cell expansion, secondary cell wall formation (involving cellulose, hemicellulose, cell wall proteins, and lignin biosynthesis and deposition) and programmed cell death (Li *et al*. 2009). These processes are strongly interlinked and modulation of any one aspect of wood formation may affect many other aspects.

To date, no Kelampayan EST information is available in the NCBI GenBank. Therefore, we applied genomics approaches to explore the molecular basis of wood formation in Kelampayan. Here we report the generation and analysis of a genomic resource (10,368 expressed sequence tags, ESTs) for wood formation in Kelampayan via high-throughput DNA sequencing of cDNA clones derived from a 2-year old developing xylem tissues.

Assembly of 6,622 high quality ESTs from 5' end sequences generated 4,728 xylogenesis unigenes with an average length of 672bp. The analysis formed 2,100 consensus contigs sequences (representing 3,994 or 60.3% of the total high quality ESTs), with a length ranging from 132bp to 2706bp and an average length of 621bp. The remaining 2,628 (representing 39.7 % of the total high quality ESTs) were singletons which ranged from 104 to 839bp, with an average length of 723bp. About 59.3% of the ESTs were assigned with putative identifications whereas 40.7% of the sequences showed no significant similarity to any sequences in the GenBank. Assembly analysis revealed a redundancy level of 28.5**%** in the Kelampayan EST database (Table 3). By comparison, the EST redundancy in the kelampayan EST database is comparable to the estimated redundancy of 28% in *Populus* (Aspeborg *et al*., 2005) and the 28.8% in *Pinus radiate* (Li *et al*., 2009).

Table 3: Assembly of Kelampayan xylogenesis ESTs from the developing xylem cDNA library

	Number	%
Total clones sequenced	10,368	
Clean ESTs for assembly	6,622	
Average EST length (base)	478	
No. of contigs	2,100	
No. of ESTs within consensus	3,994	60.3
No. of singletons	2,628	39.7
Unique sequences[a]	4,728	71.5
Redundancy[b]		28.5

[a]: Calculated as the sum of the number of contigs and singletons within a library.
[b]: Redundancy was estimated by: 1-(number of unique sequences/number of Cleaned ESTs)
EST Redundancy in Populus (Aspeborg et al., 2005) was 28% & in Pinus radiata D. Don (Li et al., 2009) was 28.8%

The most abundant protein in the ESTs whose putative function was inferred from sequence comparison was 60s ribosomal protein with 92 ESTs, followed by 40s ribosomal protein with 42 ESTs. Interestingly, most genes involved in lignin biosynthesis were present in the kelampayan EST database with 1 to 21 ESTs. These include *phenylalanine ammonia-lyase* (*PAL*), *cinnamate 4-hydroxylase* (*C4H*), *coumarate 3-hydroxylase (ρ-coumaryl shikimate/quinate 3-hydroxylase)* (*C3H*), *caffeic acid O-methyltransferase* (*COMT*), *caffeoyl-CoA-3-O-methyltransferase* (*CCoAOMT*), *4-coumarate:CoA reductase* (*4CL*), *ferulate 5-hydroxylase* (*F5H*), *cinnamyl alcohol dehydrogenase* (*CAD*), *hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyl transferase* (*HCT*) and *cinnamoyl-CoA reductase* (*CCR*). *COMT*, *CCoAOMT* and *C3H* are in the 30 highly abundant genes with 18 to 21 ESTs. Also, several ESTs exhibiting homologies to cell wall biosynthesis genes were also identified in the Kelampayan EST database. The most highly abundant cell wall genes are tubulin (42 ESTs), *arabinogalactan prtoein* (*AGPs*) genes (30 ESTs) and *cellulose synthase* (13 ESTs). Other cell wall related genes including *sucrose synthase* (*SuSy*), *expansin*, *UDP-glucose dehydrogenase* (*UGDH*), *xyloglucan endotransglycosylase* (*XET*) and *pectate lyase* are moderately abundant with 2 to 11 ESTs in the Kelampayan EST database.

This study has generated an important genomic resource for wood formation in Kelampayan. The identified genes in this study will provide a useful resource for identifying molecular mechanisms controlling wood formation and will also be candidates for association genetic studies in Kelampayan aiming at the production of high value forests (Figure 5). Furthermore, comparison of Kelampayan ESTs with sequences from angiosperms will also generate valuable information about the evolution of higher plants.

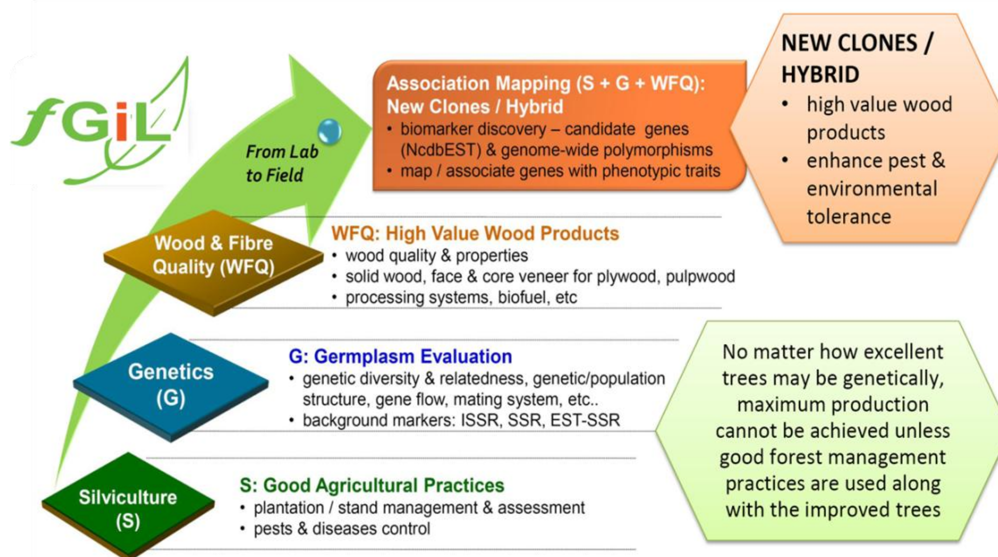


Figure 5: Genomics-based breeding or association mapping of Kelampayan

Conclusions and Future Challenges

For the world to meet the current demand for wood it needs to invest much more in the research and development of high-yielding, faster growth and short-rotation plantation forests (harvestable within 8-10 years/rotation) compared to 50-60 years/rotation. In this regard, forest biotechnology is essential in achieving this goal. Over the past 3 years, we have successfully developed an array of highly informative and polymorphic DNA markers specific for identifying the genetic makeup of two fast growing indigenous tree species, i.e. Kelampayan and Sawih; 2) the one step 'Touch-incubate-PCR' approach for preparing plant tissues for high throughput genotyping, and 3) a genomic resource database, aka CADAMOMICS (10,368 ESTs) for wood formation in Kelampayan via high-throughput DNA sequencing. These tools will greatly facilitate the selection of quality planting materials for planted forest development in Sarawak as well as long-term tree improvement activities by integrating the silviculture practices, genetics and genomics, and wood and fibre quality components into our breeding strategy or known as association mapping. The outcome that will be produced through association mapping is new clones for plantation, and also the crosses between these new clones to produce hybrid, with double yields as well as enhance pest and environmental tolerance. Such new clone or hydrid has great economic potential.

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PLENARY 3:

ROLE OF CarBa SUBUNIT IN HETERO-MULTIMERIC EXTRADIOL DIOXYGENASE CarBaBb INVOLVED IN CARBAZOLE DEGRADATION

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ABSTRACT

Extradiol dioxygenase is a type of enzyme involved in the ring cleavage (*meta*-cleavage) in the carbazole degradation pathway. Extradiol dioxygenase catalyzes the ring cleavage at the C-C bond adjacent to the vicinal hydroxy groups in a process called *meta*-cleavage. Most ring cleavage enzymes of aromatic compound degradation pathway are single protein enzymes, but the enzyme from carbazole degradation pathway consists of 2 distinct subunits, small subunit CarBa and large subunit CarBb. CarBb is homologous to other extradiol dioxygenase but the role of CarBa remains unknown. Previously, CarBb was reported to be inactive without the co-expression of CarBa. In this study, CarBb was expressed in *Escherichia coli* and purified while retaining its activity to deduce the role of subunit CarBa. Enzymatic activity was highest at temperature of 15 °C but drastically reduced at higher temperature suggesting CarBa involvement in the thermoinstability of CarBb subunit.

Keywords: Extradiol dioxygenase, *meta*-cleavage, carbazole degradation

INTRODUCTION

Carbazole, a compound produced from impurities of fossil fuel, share the same plane structure of dioxin and characterized as a recalcitrant chemical and was reported to have carcinogen properties. Isolation of bacteria capable of degrading carbazole as sole carbon and nitrogen source have been reported from both Gram-negative and -positive strains and genes responsible for degradation of aromatic compounds have been discovered. The *car* genes of *Pseudomonas resinovorans* CA10 have been studied most extensively among other carbazole degrading bacteria. Extradiol dioxygenase is a type of enzyme involved in the ring cleavage (*meta*-cleavage) in the carbazole degradation pathway. Extradiol dioxygenase of strain CA10 is a class III extradiol dioxygenase designated CarBaBb are coded by *carBa* and *carBb* genes. Although most extradiol dioxygenase are homo-multimeric, CarBaBb is made of two proteins, CarBb, a larger subunit which carries the catalytic site and CarBa, a smaller subunit which function remains unknown. The catalytic site of this enzyme is on the larger subunit and it was reported that activity was only detected when both proteins were expressed (Sato *et al*, 1997). The same were also reported for another hetero-multimeric extradiol dioxygenase LigAB of *Pseudomonas paucimobilis* SYK-1 (Noda *et al*, 1990) and FlnD1D2 of *Terrabacter* sp. DBF63 (Habe *et al*, 2004). Studies on other extradiol dioxygenases such as BphC and LigAB revealed many details on the reaction mechanism of these ring-cleaving enzymes. However, the significance of the

small subunit in a hetero-multimeric extradiol dioxygenase remains unknown. In this study, we investigated the role of CarBa subunit in CarBaBb hetero-tetrameric extradiol dioxygenase. The role of CarBa was deduced by comparison of CarBaBb characteristics with active CarBb protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and culture conditions

Escherichia coli strains JM109, DH5α (Takara Shuzo Co., Ltd., Kyoto, Japan), and BL21(DE3) (Novagene, Inc., Madison, WI) were used for expression plasmid construction and protein expression. Expression plasmids were constructed based on pET-15b. *E. coli* strains were grown on LB medium or 2 x YT medium containing appropriate antibiotics at 37˚C with shaking. Appropriate amount of isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium to induce expression. Expression plasmid harboring *carBb* gene was designated as p15CA10Bbht.

Expression and purification

E. coli BL21 (DE3) transformed with p15CA10Bbht was grown at 37 °C to at least OD_{600} 1.0 before expression was induced by the addition of IPTG. After this addition, the culture was grown at 25 °C for approximately 4 hours before harvest. Cells were later sonificated with microtip on ice. Cells extract were then centrifuged to separate soluble and insoluble fractions. Soluble fractions were used for purification. Purification procedures for CarBaBb were all conducted at 4 ˚C. Chromatography was conducted on Akta FPLC system (Amersham Biosciences). The purification was conducted in 2-step fashion, Metal chelation affinity and gel filtration. Purified protein was checked with SDS-PAGE and protein concentrations were determined using the method of Bradford.

Optimal temperature and optimal pH assay

To determine the optimal temperature for CarBaBb activity, measurements were conducted at temperatures ranging from 5 ˚C to 60 ˚C. For these tests, *meta*-cleavage activities were measured using 2,3-dihydroxybiphenyl (2,3-DHBP) (final concentration 100 µM) as substrate. For this temperature assay, 50 mM Tris-HCl buffer (pH 7.5) was used. To determine optimal pH for CarBb activity, buffers used were 50 mM citrate buffer (pH 3.0-6.0), 50 mM 2-(N-morpholino) ethanesulfonic acid (MES)-NaOH buffer (pH 5.5-7.0), 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS)-NaOH buffer (pH 6.5-8.0), 50 mM Tris-HCl buffer (pH 7.5-9.0) and 50 mM glycine-NaOH buffer (pH 8.5-10.5). Temperature 25 ˚C was maintained during meta-cleavage measurements.

RESULTS

Expression and purification of CarBb

CarBb was expressed under the influenced of *T7lac* promoter from pET vector system. *E. coli* BL21(DE3) harboring vector p15CA10Bbht showed no band corresponding CarBb enzyme at growth temperatures 30 ˚C and 37 ˚C. Further investigation revealed that at these temperatures, expressed CarBb collected mostly in the insoluble fraction of the cell extract. To obtain more enzymes in the soluble fraction, growth conditions were altered to express CarBb at a lower temperature. Active CarBb enzyme was partially recovered when expression was induced at 25˚C. The first step of purification was performed using affinity chromatography. CarBb protein was eluted as a single dominant peak. Similar results were also obtained for the second step of

purification using gel filtration. CarBb was purified to homogeneity judging from SDS-PAGE shown in figure 1. Summary of purification is shown in table 1.

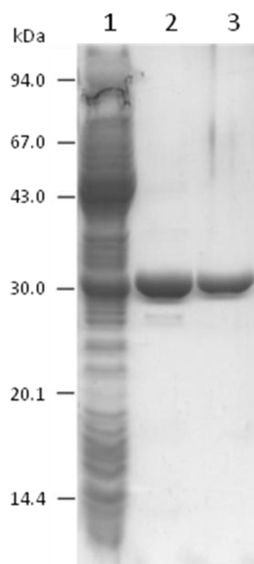

Figure 1. SDS-PAGE (15%) result showing each step of purification. Lane 1: crude cell extract, 2: CarBb after affinity chromatograpy and 3: CarBb after gel filtration.

Table 1. Summary of purification steps for CarBb from 1 l of culture.

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (10^{-2} U/mg)	Purification fold
Cell extract	60	1402	136	9.7	-
Affinity chromatography	9	53	21	39.7	4.1
Gel filtration	9	44	28	63.6	6.5

One unit (1 U) of activity was defined as the amount of enzyme needed to form 1 µmol of *meta*-cleavage compound per minute at 25 °C.

Effects of temperature and pH on activity of CarBb

Effects of temperature and pH on the activity of CarBb was tested over a wide range of conditions. pH effects were also tested using different types of buffers to ensure enzymatic activatities not biased towards any single buffer. Enzymatic activity was highest when reaction occurred at the temperature of 15 °C (Fig. 2). In comparison to CarBaBb, the maximum relative activity for CarBb was shifted 20 °C to the left suggesting weaker stability. The relative activity was below 50% after the temperature 25 °C. For pH, the ring-cleavage activity was highest at pH 7.5 and the activity was below 50% when reaction occurred outside of pH 7-8 range (Fig. 3.). Without the CarBa, CarBb tolerence towards wider pH range was reduced relative to CarBaBb.

CarBb retained full activity even after incubation up to 35 °C for 30 min but lose activity completely after incubation above 40 °C for 30 min.

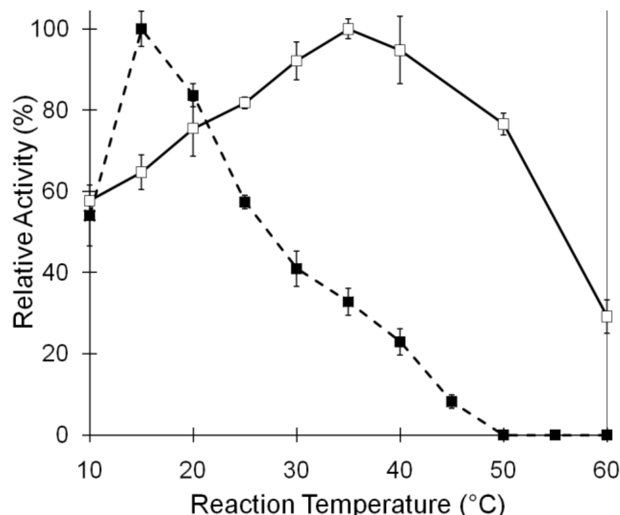

Figure 2. Enzymatic activities at various temperatures. Dotted line represent the activities of CarBb and solid line repesents activities of CarBaBb. Activity was measured in 50 mM Tris-HCl pH 7.0 buffer using 100 µM 2,3-DHBP.

DISCUSSION

In this study, only the large subunit CarBb of the 2 subunit extradiol dioxygenase enyme was cloned, expressed, purified and characterized. Although the study of CarBaBb of strain CA10 was already reported (Iwata *et al*, 2003), in this study, the characterization of only CarBb subunit provided insights to the function of small subunit CarBa which is still unknown. This study dispprove the previous report that expression of both small and large subunit of extradiol dioxygenase are indispensable for ring-cleavage activity (Sato *et al*, 1997). The active site containing Fe ion coordinated by well conserved residues which is needed for *meta*-cleavage activity are located at CarBb, thus it is not impossible for the large subunit to retain its activity by itself. It can be concluded that CarBa do not play a role in the mechanism of *meta*-cleavage.

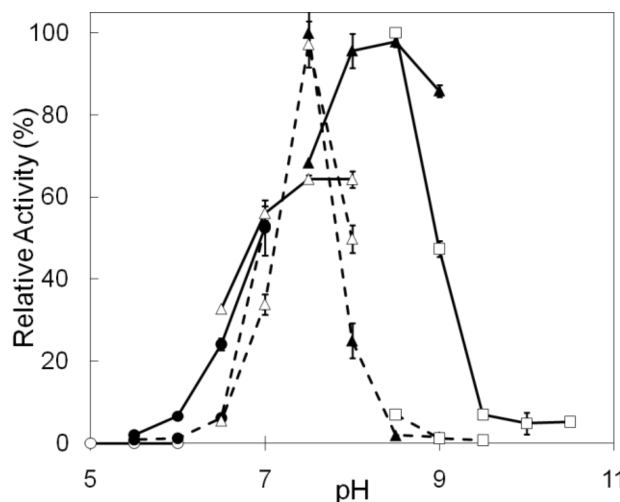

Figure 3. Enzymatic activities at various pH. Activities were measured at 25 °C buffer using 100 µM 2,3-DHBP. Buffers used were 50 mM citrate (○), 50 mM 2-(N-morpholino) ethanesulfonic acid (MES)-NaOH (●), 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS)-NaOH (Δ), 50 mM Tris-HCl (▲) and 50 mM glycine-NaOH (□).Dotted line represent the activities of CarBb and solid line repesents activities of CarBaBb.

Effects of temperatures and pH suggested CarBb to be greatly unstable when compared to CarBaBb. However, the instability of CarBb did not cause it to denature as purification was possible from soluble fractions. The instability of CarBb may cause conformational changes which affected its enzymatic activity. This study showed that CarBa is crucial for maitaining stability CarBb but did not rule out other possible roles of CarBa such as interactions with substrates. It would also be interesting to assess the influence of CarBa towards the oxidation state of Fe ion, which is essential for *meta*-cleavage activity.

In conclusion, we have shown that CarBb can function as an active *meta*-cleavage enzyme independent of its small subunit CarBa. Nevertheless, enzymatic activity was more efficient when both CarBa and CarBb is expressed. The role of CarBa in maintaining the stability of CarBb was also shown.

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PLENARY 4:

DISTRIBUTION AND DYNAMICS OF INDIGENOUS HYDROCARBON DEGRADING FUNGI AND THEIR ENZYMES

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ABSTRACT

A total of eighteen indigenous hydrocarbon degrading fungi isolates that grows well on minimal salts medium supplemented with 1% (v/v) crude oil has been successfully isolated and characterized morphologically and molecularly from the oil, soil and water of oil contaminated sites in Kota Samarahan, Pending, Kuching, Lutong, Miri, Bintulu and Brunei Darussalam. Most of the hydrocarbon degrading fungi isolates obtained are from the genus of *Trichoderma spp*, *Penicillium spp* and *Aspergillus spp*. The main enzymes secreted by the fungi during the hydrocarbon degradation are the peroxidases namely lignin peroxidase, manganese peroxidase & laccase. Biodegradation trials were performed on minimal salt medium amended with 1% (v/v) crude oil. Gravimetric analysis and toluene method were carried out to determine the hydrocarbon degradation activity quantitatively. The selected fungal isolates were shown capable of degrading both the aliphatic and aromatic compounds in the crude oil.