CLONING AND CHARACTERIZATION OF THE GENE ENCODING FOR COPPER/ZINC SUPEROXIDE DISMUTASE FROM LYMPHATIC FILARIASIS

A THESIS

BY

PIYAPA KIATSOMCHAI

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โคลนนิ่งและศึกษายีนคอปเปอร์/ซิงค์ซูปเปอร์ออกไซม์ดิสมิวเทสในพยาธิเท้าช้าง

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เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา
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โรคเท้าช้าง เป็นปัญหาทางสาธารณสุขในประเทศกำลังพัฒนาหลายๆ ประเทศ ซึ่งองค์การอนามัยโลก (WHO) ต้องการกำจัดโรคนี้ให้หมดไปภายในปี ค.ศ. 2020 ถึงแม้ว่าอัตราความชุกของการติดเชื้อ *Wuchereria bancrofti* จะมีน้อยมากในประเทศไทย แต่การอพยพของแรงงานชาวพม่าเข้ามาทำงานในประเทศไทย เป็นสาเหตุหนึ่งของการเพิ่มอุบัติการณ์ของโรคนี้ ปัจจุบันมีการใช้ยา ivermectin และ diethylcarbamazine (DEC) ในการรักษาโรคเท้าช้าง ซึ่งต้องใช้ติดต่อกันเป็นเวลานานจึงจะเห็นผล อย่างไรก็ตามพบว่ายาดังกล่าวมีผลข้างเคียง และยังมีรายงานการพบเชื้อดื้อยา ivermectin อีกด้วย ดังนั้นการศึกษายีนใหม่ๆ จึงเป็นทางเลือกหนึ่ง ที่สามารถนำมาเป็นเป้าหมายของการรักษาโรคเท้าช้างด้วยยา ยีนซูปเปอร์ออกไซด์ ดิสมิวเทส (Superoxide dismutase, SOD) สามารถผลิตเอนไซม์ SOD ในกระบวนการเมตาโบลิสมที่มีความสำคัญในการลดระดับซุปเปอร์ออกไซด์ ซึ่งเป็นสารพิษในเซลล์ของพยาธิ ดังนั้นการยับยั้งการทำงานของเอนไซม์นี้จะมีผลในการยับยั้งการเจริญเติบโตของตัวพยาธิด้วย งานวิจัยนี้ได้ทำการแยกยีน *Cu/Zn SOD* จากไมโครฟิลาเรียของ *W. bancrofti* (*Cu/Zn WbSOD*) ด้วยวิธี PCR โดยใช้ degeneracy primers เพิ่มชิ้นส่วนของยีน จนกระทั่งได้ลำดับเบสของยีนครบ พบว่า *Cu/Zn WbSOD* ประกอบด้วย 1037 นิวคลีโอไทด์ โดยมีลำดับเบสเฉพาะส่วนที่เป็นยีนและส่วนที่ไม่ใช่ยีนจำนวน 4 exon (477 นิวคลีโอไทด์) และ 3 intron ตามลำดับ เมื่อเปรียบเทียบลำดับเบสของ *Cu/Zn WbSOD* กับยีน *Cu/Zn SOD* ในสิ่งมีชีวิตอื่นๆ ในกลุ่มเดียวกันพบว่ามีความเหมือนอยู่ระหว่าง 63-96 % ข้อมูลเปรียบเทียบลำดับกรดอะมิโนของ Cu/Zn WbSOD กับ intracellular Cu/Zn SOD ทั้ง 5 ชนิด ในสปีชีส์ต่างๆ พบว่า มีความเหมือนอยู่ระหว่าง 66-96% นอกจากนี้ยังแสดงให้เห็นถึงบริเวณอนุรักษ์ (conserved) ของบริเวณเร่งและบริเวณจับจำเพาะของเอนไซม์ เมื่อเปรียบเทียบข้อมูลของลำดับเบสและกรดอะมิโนของยีน *Cu/Zn WbSOD* ในคน พบว่ามีความเหมือนอยู่ที่ 48 % และ 56 % ตามลำดับ และจากการหาความสัมพันธ์ทางวิวัฒนาการพบว่ายีน *Cu/Zn WbSOD* มีความสัมพันธ์ใกล้เคียงกับยีน *Cu/Zn WbSOD* ที่พบใน *Brugia malayi* และ *B. pahangi* ซึ่งข้อมูลดังกล่าวจะเป็นประโยชน์สำหรับการออกแบบยาที่สามารถยับยั้งการทำงานของเอนไซม์ Cu/Zn WbSOD ในกลุ่มของพยาธิเท้าช้าง

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AN ABSTRACT

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Lymphatic filariasis is a significant health problem in many developing countries. The World Health Organization (WHO) has launched a program to eliminate lymphatic filariasis by the year 2020. Although the prevalence of *Wuchereria bancrofti* infection in the Thai population is very low, the migration of Myanmar labors to Thailand could increase the incidence of bancroftian filariasis. Generally, diethylcarbamazine (DEC) and ivermectin have been proved to be effective producing suppression of microfilaraemia in lymphatic filariasis. However, both antifilarial agents generate side effects and are time consuming for its effectiveness. Hence, the repeat dosing of each drug is manipulated for filariasis treatment which may lead to the drug resistance in the future. Many researches have been focused on investigation of potential alternative genes or enzymes as the drug targets for the treatment. This study has emphasized on isolation and characterization of superoxide dismutase gene from lymphatic *W. bancrofti* due to its important role in destruction of the cell toxic agent superoxide. The inhibition of this enzyme activity leads to the increase of superoxide in the cell and finally causes the cell-death of parasite. The *Cu/Zn SOD* gene from *W. bancrofti* (*Cu/Zn WbSOD*) was isolated by PCR using degeneracy primers. The complete *Cu/Zn WbSOD* gene consisted of 1,037 nucleotides containing 4 exon (477 nucleotides) and 3 intron regions. The nucleotide sequence comparison of *Cu/Zn WbSOD* gene against those from other organisms showed homology 63-96%. The *Cu/Zn WbSOD* gene explicited a 48% similarity to that of human with 56% similarity in amino acid sequences. The Cu/Zn WbSOD enzyme showed 66-96% homology to 5 Cu/Zn SOD from other species with all of the binding sites and active sites were conserved. The molecular phylogenetic analysis of *Cu/Zn WbSOD* gene against those from other organisms revealed that the gene was closely related to those of filarial *Brugia malayi* and *B. pahangi.* The data are beneficial for the design of anti-filarial drug based on the inhibition of Cu/Zn SOD metabolic enzyme.

The thesis titled

" Cloning and Characterization of the Gene Encoding for Copper/Zinc Superoxide Dismutase from Lymphatic Filariasis "

by

Piyapa Kiatsomchai

has approved by The Graduate School as partial fulfillment of the requirements for the Master of Science in Molecular Biology of Srinakharinwirot University.

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CHAPTER I

INTRODUCTION

Lymphatic filariasis or elephantiasis, the mosquito-borne disease, is the major public health problem that infected over billion people in over 80 countries. Among them, over 120 million have already been affected and over 40 million are seriously incapacitated and disfigured. The clinical manifestation of infection is elephantiasis, genital swelling and tropical eosinophilia. The lymphatic filariasis is abundant and widely spreads in the tropical and subtropical areas where the prevalence of infection is continuing to increase. Lymphatic filariasis is mainly caused by three types of parasitic worms, *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. Refering to previous reports, *W. bancrofti* is widely distributed in endemic regions i.e. Africa, India, the Pacific islands, South America and Southeast Asia whereas *B. malayi* is mainly distributed in Asia countries such as China, Korea, India, Indonesia, Malaysia, Philippines and Sri Lanka. At present, *B. timori* is found only in Indonesia, especially at the islands of Alor, Flores, Timor.[1-5] In Thailand, only two types of parasitic worms, *W. bancrofti* and *B. malayi* are reported.[6-7]

At present, diethylcarbamazine citrate (DEC) and ivermectin are the only drugs that have been used for filariasis treatment. DEC has been the major drug used since 1947. Its activity is primarily directed against blood-born microfilariae (mf) of which the parasite stage is ingested by the mosquito vector. In a long term treatment, DEC exhibits the lethal effect on adult worms. However, DEC causes the side effect and is time consuming for its effectiveness as repeat doses.[8-12] Alternatively, Ivermectin, 22, 23-dihydro derivative of avermectin B1, becomes available for treatment of lymphatic filariasis since it inhibits the activity of glutamate-gated chloride channel in the central nervous system (CNS) causing the paralysis of the microfilaria. Ivermectin is very effective in immediately clearance of blood circulated microfilaria. Unfortunately, ivermectin showed no effect on adult worms. Ivermectin is widely used as it is active at extremely low dosage against a wide variety of nematodes and arthropod parasites such as *Onchocerca volvulus*, *B. malayi* and *W. bancrofti*. The low-dose of ivermectin (25 μg/kg), despite equivalent efficacy in parasite killing, has minimal clinical reaction scores

and are not correlated with parasitemia.[13-18] However, the high dose of ivermectin has been proved to be effective than the low dose.[19-21] Recently, the presence of an ivermectin-resistant organism is reported and confirmed in South Africa.[22] According to the WHO meeting in 1998, the new alternative drug targets have been focused for microfilaricides to combat any emergence of ivermectin resistance and the side effect of DEC.[23] Basically, the new drugs must be safe, curative, and effective in few doses as well as they must be cheap and chemically stable. The genes encoding for metabolic pathways that are essential for parasite growth are the main targets for investigation. Ideally, the criteria for selection of target genes are relied on the significantly different in nucleotide sequences in comparison to the similar gene of host or the target genes are existed in parasite but not in the host. Based on these criteria, superoxide dismutase (SOD) is one of the potential metabolic enzymes that may play an important role as the new drug target due to its activity in protection of parasite from the host response and eliminate the cell toxic agent superoxide. Inhibition of enzyme activity leads to the destruction of the parasite. Investigation and characterization of Cu/Zn SOD will be beneficial for the design of the anti-filarial drugs.

CHAPTER II

LITERATURE REVIEW

1. Human lymphatic filariasis

Lymphatic filariasis, the mosquito-borne disease, is mainly caused by three types of parasitic worms, *W. bancrofti*, *B. malayi* and *B. timori.* Among them, ninety percent of lymphatic filariasis infections are caused by *W. bancroft* which is widely distributed in endemic regions such as Africa, India, the Pacific islands, South America and Southeast Asia including the west of Thailand. *B. malayi* is widely distributed in Asia countries such as China, Korea, India, Indonesia, Malaysia, Philippines, Sri Lanka and the south of Thailand, *B. timori* infection occurs in Indonesia at islands of Alor, Flores, Timor.[4-5] (Figure 1) The adult worms actually live in the lymphatic vessels near the lymph nodes where they distort the vessels and cause local inflammation. In advanced stages, the worms can actually obstruct the vessels, causing the surrounding tissue to become enlarged. Bancroftian filariasis caused by *W. bancrofti* affects the legs and genitals; where as, the Malayan filariasis caused by *B. malayi* affects the legs below the knees. Repeated episodes of inflammation lead to blockage of the lymphatic system, especially in the genitals and legs. This causes the affected area to become grossly enlarged, with thickened and coarse skin which is called elephantiasis.[2,24]

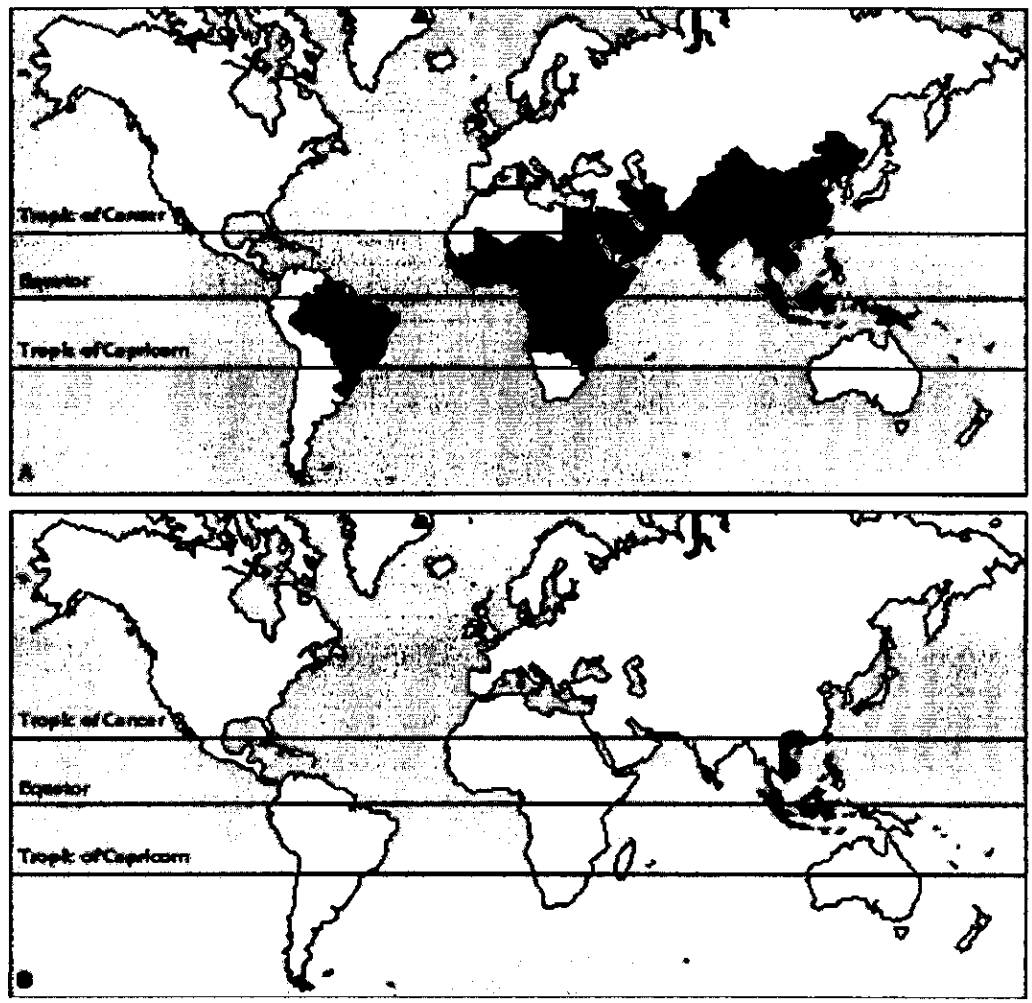


Figure 1 Distribution of *W. bancrofti* (**A**), *B. malayi* and *B. timori* (**B**).[25]

1.1 Taxonomy

Human filariasis is classified as indicated belowed.[5]

Phylum	Nematoda
Class	Secernentea
Subclass	Spiruria
Order	Spirurida
Suborder	Spirurina
Superfamily	Filarioidea
Family	Filariidae
Genus	*Wuchereria* sp., *Brugia* sp.
Species	*Wuchereria bancrofti, Brugia malayi*

1.2 Morphology

The adult worm morphology of *W. bancrofti* is white and threadlike. The female worms have 80 to 100 mm in length and 0.24 to 0.30 mm in diameter; whereas, the males are about 40 mm by 0.1 mm. Adults produce microfilariae and are sheathed. Their size is 244 to 296 μm by 7.5 to 10 μm. The sheath, actually the egg shell, is very thin and delicate that can be stained by haematoxylin. It is not lost when circulates in the host blood but is digested away in the stomach of mosquito.[26-27] (Figure 2)

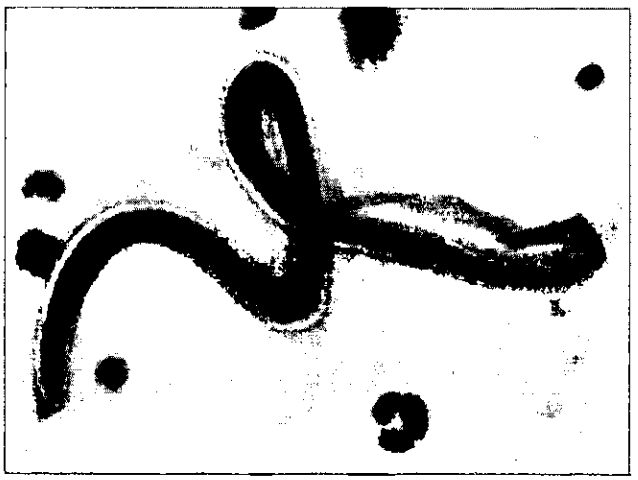


Figure 2 Haematoxylin stained of *W. bancrofti* microfilaria. Its body is sheathed and gently curved. Its tail is tapered to a point. The nuclear column (the cells that constitute the body of the microfilaria) is loosely packed. The nuclei can be visualized individually and do not extend to the tip of the tail. (Haematoxylin stained x400)[27]

1.3 Life cycle

The life cycle of *W. bancrofti* has been discovered by Manson since 1878. The transmission by mosquitoes was the first demonstrated using arthropod as vector of parasitic organism. The mosquito that was found by Manson in China was *Culex fatigans*. Laterly, it has been found that various species of *Aedes* and *Anopheles* are also the important vectors in some other parts of the world. The life cycle of *W. bancrofti* is shown in Figure 3. The larva infections stage, L3, of microfilariae enter human through the puncture site in the skin when the mosquito takes a blood meal. Within one year, the microfilariae migrate to the lymphatic system, mature into adult worms, mate and produce more microfilariae. The adult worms live in the vessels of the lymphatic system, especially in the legs, arms, scrotum and breasts. They cause the vessels to dilate so that the lymph fluid moves slowly and ineffectively. When bacteria build up and cannot be removed, the vessels can become inflamed. Adult worms can live for 4-6 years and produce millions of microfilariae that are released in the lymph and travel into blood circulation via the thoracic duct. Microfilariae are sheathed, circulate in the

peripheral blood, and show marked periodicity that corresponds to the biting habits of the mosquito vector. In most areas, *W. bancrofti* is nocturnal periodicity with the highest concentrations of microfilariae in the blood happening around midnight. After ingestion, the microfilariae lose their sheaths and some of them pass through the wall of the proventriculus and cardiac portion of the mosquito's midgut and reach the thoracic muscles into first-stage larvae (L1) and second-stage larva (L2) and subsequently into third-stage (L3) infective larvae for 2 weeks. The third-stage infective larvae migrate through the hemocoel to the mosquito's prosbocis and can infect another human through the puncture site in the skin when the mosquito takes a blood meal.[24,26,28]

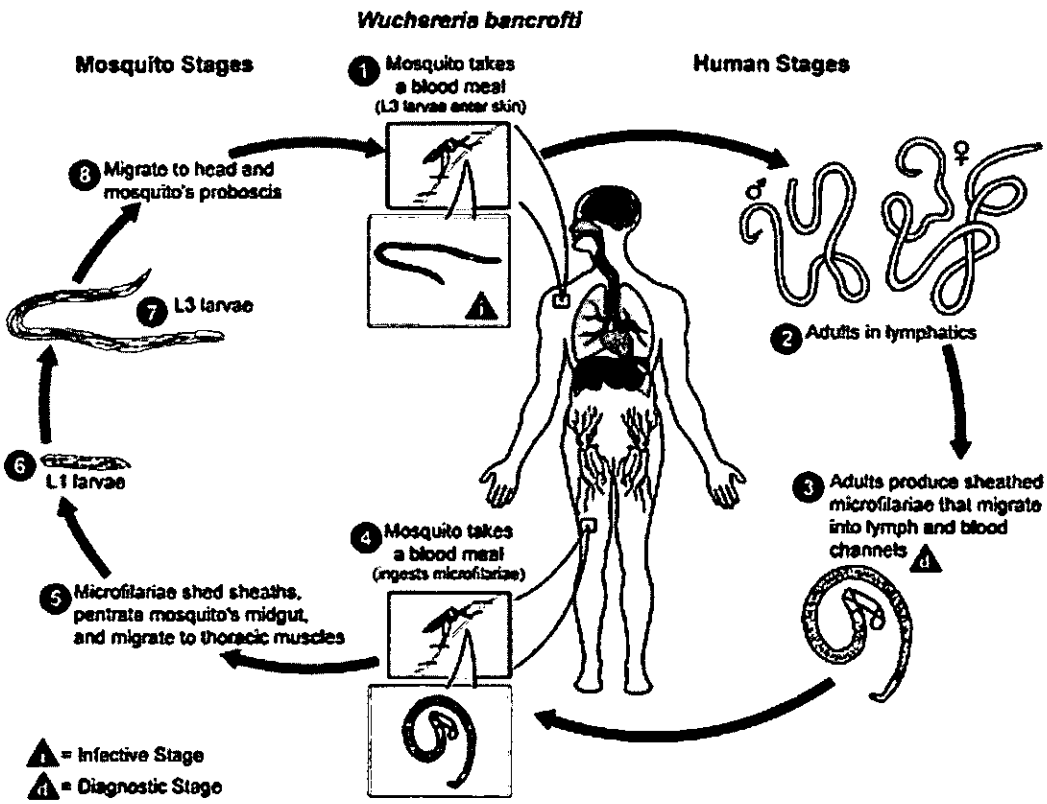


Figure 3 Life cycle of *Wuchereria bancrofti.* [29]

1.4 Epidemiology

W. bancrofti is abudant in many parts of the tropics and subtropics. Its vectors which are the common house mosquito contain three genera including *Culex pipiens quinquefasciatus (C. fatigans)* in many urban centers, *Aedes* species in the South Pacific islands, and *Anopheles* species in more isolated rural areas. Human infection is closely related to the ecology of the mosquito vectors as well as to human habits. For instance, the occurrence of periodic filariasis in areas of dense population and poor sanitation is paralled to the distribution of its principal vector, *C. fatigans*, which breeds in sewage-contaminated water.[28]

Previously, humans have been assumed to be the only hosts for *W. bancrofti*. At present, several species of monkeys have been experimentally prooved to be the possible host of *W. bancrofti*. However, no naturally infected reservoirs other than humans have been identified.[30]

1.5 Periodicity

In most parts of the filarial endemic area in the world, they are periodic form. Microfilariae present in very small numbers in the circulating blood during the daytime hours which is usually undetectable and their appearance at greatest density at night generally between the hours of 10 PM and 2 to 4 AM. (Figure 4) The subperiodic form of filariasis occurs throughout the pacific islands. Persons infected with this strain exhibit microfilaremia at all times, but the organisms are present in greatest numbers between noon and 8 PM. The subperiodic form has also been found in some areas of Vietnam. The suggestion has been made that the subperiodic form is recognized as a separate variety, *W. bancrofti* var. *pacifica*, or that it should form a distinct species, *W. pacifica*. Neither proposal has been widely accepted, as the morphologic differences reported between adult worms of the periodic and nonperiodic forms are extremely minor, and microfilariae of the two sorts appear to be identical.

Microfilarial periodicity is obviously survival value because it enhances the opportunity for microfilariae to be ingested by insect vectors at certain times. Generally, the surge of microfilariae coincides with the active feeding periods of the various insect vectors. For example, the insect vector for the nocturnal *W. bancrofti* strain is the nocturnally feeding mosquito, *C. fatigans*, while the vector for the subperiodic strain is the diurnal feeder, *Aedes polynesiensis*.

2. Filariasis in Thailand

There are two species of lymphatic filaria in Thailand, *W. bancrofti* and *B. malayi*. *W. bancrofti* is found in northern and western parts whereas *B. malayi* is predominant in the southern regions of the country. Brugian filariasis is caused by *B. malayi*. The two forms of *B. malayi*, periodic and subperiodic forms, are mostly found in the flat and low-lying areas around the coastal belt in the southern part of Thailand. The subperiodic form of *W. bancrofti* is abundant in hilly, forested and mountain areas in the western part of Thailand.[32] (Figure 5) Bancroftian filariasis caused by nocturnally subperiodic *W. bancrofti* (rural strain) has been distributed along the Thai-Myanmar border from Mae Hong Son to Ranong Provinces since 1970.[6-7,33]

The dynamic epidemiology of *W. bancrofti* may have been occurred through migration of infected Myanmar laborers to Thailand. During 1993-1997, more than 700,000 Myanmar laborers have migrated in the big cities where the potential vector is abundantly available.[33] Previous report has shown that approximately 2-5% of them are carriers of nocturnally periodic *W. bancrofti* (urban strain).[34]

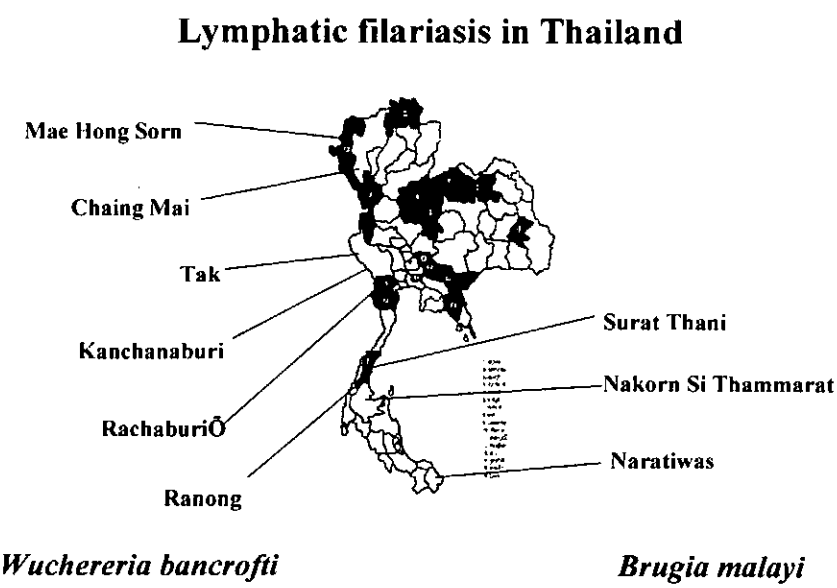


Figure 5 The distribution of lymphatic filariasis in Thailand.

3. Treatment of lymphatic filariasis

To date, diethylcarbamazine citrate (DEC) and ivermectin are the only two common drugs that have been used for filariasis treatment. Many experiments have described and demonstrated the efficacy of each drug as well as the combination of both drug to other nematocidal agent e.g. albendazole to control the disease. However, the use of DEC and/or ivermectin has their own limit as described belowed.

3.1 Diethylcarbamazine citrate (DEC)

DEC has been the major drug used since 1947. Its activity is directed primarily against blood-borne microfilariae (mf), the parasite stage ingested by the mosquito vector.[8,10] DEC can effectively reduce the microfilarial load but has more limited effect on the adult worm.[11,12] The microfilaricidal action of DEC is unclear.[35] In vitro studies have shown that DEC stimulates the platelet to release underfined (Ox)-type free radical species, the probable mechanism by which microfilaria are killed.[36] The drastic morphological damage to microfilariae of *W. bancrofti* after treatment *in vitro* and *in vivo* with DEC has been detected indicating a direct mode of action of this major anti-filarial drug. One of the first morphological alterations produced by treatment with DEC is the loss of microfilarial sheath and lysis of the cytoplasm, with the destruction of all organelles, numerous large vacuoles and nuclear condensation.[37-39]

DEC is generally administered at the rate of 6 mg/kg of body weight. In the past, it was usually given either daily for 12 days, or once a month for a year. Comparable results are obtained by the use of a single annual dose which was more practical for mass treatment programs. With all such treatment programs, microfilariae disappear from the blood of most patients within 2 weeks and reappear in smaller numbers within 3 to 6 months. On the basis of parasite antigen levels before and following treatment, it is suggested that DEC administered at 6 mg/kg body weight daily for 12 days has approximately a 50 percent macrofilaricidal effect. However, the once-yearly administration has been tested to be a lesser impact on the adult worms. A long-term suppression of microfilaremia has been achieved by the concurrent administration of DEC plus ivermectin on the same once-yearly dosage schedule.[26]

In spite of its effective in filariasis treatment, DEC causes the side effect and is time consuming for its effectiveness as repeat doses. DEC as a number of post treatment adverse-reaction syndromes are induced by the very rapid killing of microfilariae. These include the

following: (1) General malaise with headache, weakness, joint pains, anorexia, nausea, and vomiting; (2) Dermal and systemic effects (especially in patients with onchocerciasis, in which the response has been termed the Mazzotti reaction) caused by an acute inflammatory response following clearing /killing of microfilariae and characterised by itching and swelling of the skin, fever, tachycardia, hypotension, adenitis, and severe inflammatory reactions in both the anterior and posterior segments of the eyes of patients with onchocerciasis who have ocular infection; and (3) Encephalopathy in patients being treated with DEC for *Loa loa* infection.[40-42]

3.2 Ivermectin

Ivermectin, a macrocyclic lactone antibiotic belonging to the group of avermectins derived from *Streptomyces avermitilis*. This drug has been used extensively for the control of a wide variety of parasites in farm and domestic animals. In humans, ivermectin has been used extensively to control onchocerciasis in endemic countries of Africa and Latin America since 1987. This drug is also effective in the treatment of other filariasis such as loiasis and bancroftian filariasis and other intestinal nematodes.[26,43-46] The effect and tolerance of ivermectin are considered to be about the same in all cases of filariasis due to its efficacy on microfilaria, but not adult filaria.[18] The nematocidal drug ivermectin is believed to kill worm by opening a glutamate-gated chloride channel (AVR-15) on pharyngeal muscle, causing complete pumping inhibition.[47] Dent *et al.* (2000) proposed another model in which ivermectin also killed worms by inhibiting pumping. In this model, ivermectin irreversibly opened a glutamate-gated chloride channel composed of *avr-14* and/or *avr-15* in an extrapharyngeal neuron. This resulted in chloride influx and neuron hyperpolarization which descended to the pharynx via gap junctions encoded by *unc-7* and inhibited pumping.[48] (Figure 6)

According to many publications on the use of ivermectin for treating lymphatic filariasis, the single dose of 400 μg/kg has been examined to show maximal responses, but a number of practical considerations suggest that either 400 μg/kg or 200 μg/kg doses would be acceptable for use in lymphatic filariasis control programs. However, the combination of DEC and ivermectin are preferable because of the macrofilaricidal properties of DEC. For mass treatment programs, the continuing yearly drug treatment for at least 4 years has been recommended.[26,40]

The reports of resistance to ivermectin in nematodes are increasingly common. Ivermectin resistant organism was reported and confirmed in South Africa. The original helminth fauna of sheep from this Mediteranean –like climate is composed mainly of species from *Ostertagia*, *Trichostrongylus* and *Nematodirus*, with *Haemonchus* being less common.[22] In addition, ivermectin resistances occur in nematodes of sheep and goats whereas closantel resistance has been found in *Haemonchus contortus*.[49] Recently, the presence in the nematode *Caenorhabditis elegans*, simultaneous mutation of three genes, *avr-14*, *avr-15*, and *glc-1*, encoding glutamate-gated chloride channel (GluCl) α-type subunits, led to high-level resistance to ivermectin.[48]

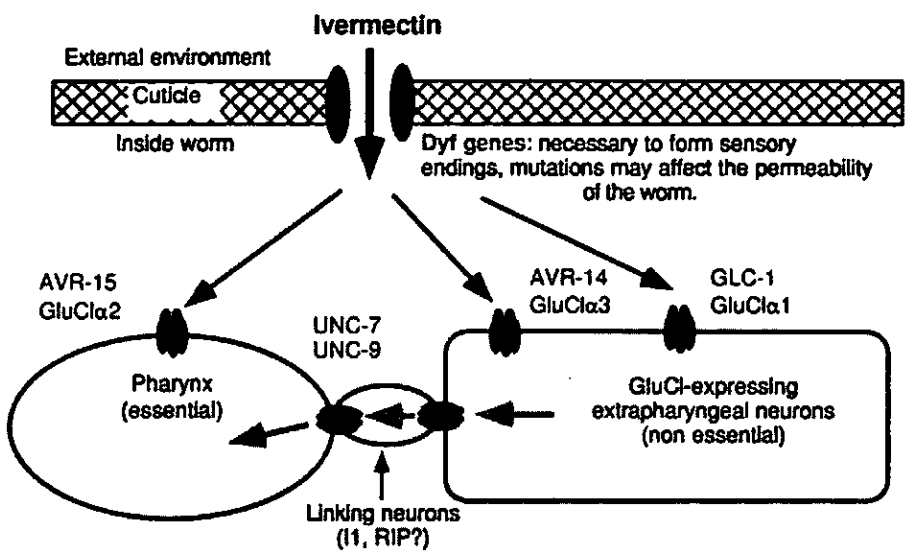


Figure 6 Preposed model to describe mechanisms of ivermectin activity. Black arrows indicated the diffusion of ivermectin and the gray arrows indicated the flow of ivermectin-induced hyperpolarizing potential.[48]

4. superoxide dismutases (SOD)

Due to the emergence of ivermectin resistance and the side effect of DEC described previously, the alternative new drug targets have been focused for microfilaricides. Among them, superoxide dismutase (SOD) is one of the metabolic enzyme that may play an important role as the new drug target.

Superoxide dismutase (SOD) was first isolated by Mann and Keilis in 1938 and thought to be a copper storage protein. Subsequently, the enzyme was identified by a number of names, erythrocuprein, indophenol oxidase, and tetrazolium oxidase until its catalytic function was discovered by McCord and Fridovitch in 1969. SOD is an ubiquitous metalloenzyme (E.C.1.15.1.1) in various organisms. It catalyzes the dismutation of deleterious O^{-}_{2} radicals to reduce oxidative stress in the cell. The enzyme scavenges superoxide by catalyzing its dismutation to oxygen and hydrogen peroxide as followed:

$$2O^{-}_{2} + 2H^{+} \xrightarrow{SOD} O_2 + H_2O_2$$

SOD converts superoxide radicals to a less toxic Reactive Oxygen Species (ROS), hydrogen peroxide (H_2O_2). The superoxide radical ($O_2^{-}\cdot$) is formed in the mitochondria as a by product of electron transport. They are very dangerous to the cell because they steal electrons from neighboring molecules, start a cascade of electron stealing, and finally damage the cell. In the process of removing superoxide free radicals, SOD rarely operates alone. It requires the enzyme catalase and glutathione peroxidase for next step processed to nontoxic by-products.

Catalase is located within the peroxisomes and cytosol of the cell and decomposes hydrogen peroxide to water and oxygen as followed:

$$2\ H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

Glutathione peroxidase presented primarily as a selenium containing metallo-enzyme is located within cellular membranes where it can react directly with hydrogen peroxide or with lipid hydroperoxide using reduced glutathione as its source of electrons. The enzyme glutathione peroxidase degrades the hydrogen peroxide as followed:

$$H_2O_2 + 2GSH \xrightarrow{\text{Glutathione peroxidase}} GSSG + 2H_2O$$

The metallo-enzyme superoxide dismutase comprised of metal ions in three forms referring to manganese-SOD (Mn-SOD), iron-SOD (Fe-SOD) and copper/zinc-SOD (Cu/Zn-SOD).[50-53]

Iron SOD (Fe SOD)

Fe SOD is found both in prokaryotes and eukaryotes. In eukaryotes, the enzyme has been isolated from *Euglena gracilis* and higher plants. Fe SOD is inactivated by H_2O_2 and is resistant to potassium cyanide (KCN) inhibition. There are two distinct groups of Fe SOD. The first group is a homodimer formed from two identical 20 kDa subunit proteins, with 1–2 gram atom of iron in the active centre. The second Fe group, found in most higher plants, is a tetramer of four equal subunits with a molecular weight of 80–90 kDa. Members of this group contain 2–4 gram atoms of iron in the active centre. Proteins in this group have been isolated from three prokaryotes, *Mycobacterium tuberculosis*, *Thermoplasma acisophilum*, and *Methanobacterium bryantii* in addition to one eukaryote, *Tetrahymena pyriformis*.[50-51,54-57]

Manganese SOD (Mn SOD)

Mn SOD occurs in mitochondria and peroxisomes of the plant cells. Mn SOD carries only one metal atom per subunit. The enzyme cannot function without the Mn atom present at the active site. Mn SOD is either a homodimeric or a homotetrameric enzyme with one Mn (III) atom per subunit. The enzyme is not inhibited by KCN or inactivated by H_2O_2 and is presented in both eukaryotes and prokaryotes.[54]

Copper/zinc SOD (Cu/Zn SOD)

Whereas Fe and Mn SODs are presented both in prokaryotic and in eukaryotic organisms, Cu/Zn SOD has been found mostly in eukaryotes. However, Cu/Zn SOD has been demonstrated in some bacteria, such as *Photobacterium leiognathi*, *Caulobacter crescentus*, and pseudomonads.[54] The *Cu/ZnSOD* gene is located on human chromosome 21q22.1. The gene is presented in a single copy per haploidè genome and spans 11 kb of chromosomal DNA. The coding region contains five exons interrupted by four small introns.[58]

Two different groups of Cu/Zn SOD are composed of cellular and extracellular enzymes. The human cytosolic enzyme has a dimer structure which each subunit has a molecular weight of 16 kDa that contains one ion each of copper and zinc. This enzyme is responsible for removing toxic superoxide radicals generated by a number of oxidative metabolisms. An extracellular from of Cu/Zn-SOD has been recently found in mamalian body fluids and also in some tissues. This secretory enzyme is a tetrameric glycoprotein with 30 kDa monomeric subunits. Each of secretory enzyme contains copper and Zinc in the ratio of 1:1. The extracellular form is proposed to be glycosylated through an N-linkage with a putative 18 amino acid signal peptide that is cleaved upon secretion and a hydrophilic, positively charged carboxylic terminus that is postulated to bind to heparin-sulphate receptors on cell membrane. Extracellular Cu/Zn SOD shares sequence homology with cytosolic Cu/Zn SOD in the active site but diverges in the sequence coding for subunit content. Both forms of Cu/Zn SOD are related and have been derived from a common ancestor which is different from of Mn SOD and Fe SOD.[59-65]

The *Cu/Zn SOD* nucleotide sequences from many helminth parasites have been reported. (Table 1) In 1988, the gene encoding for extracellular Cu/Zn SOD enzyme of *S. mansoni* spans 5.1 kb of DNA and possesses three exons with two introns. The encoded amino acid sequences have 39% homology to the human extracellular Cu/Zn SOD.[66] The cytosolic Cu/Zn SOD of *S. mansoni* shows 60-65% homology to 19 cytosolic Cu/Zn SOD from other species with all of the Cu/Zn binding sites and activity sites are conserved. The cDNA encodes for cytosolic Cu/Zn SOD of *S. mansoni* is approximately 600 bp in length which is corresponded to a protein of 153 amino acids with a predicted molecular mass of 15,693 Da.[67] In 2000, the molecular mass of cytosolic Cu/Zn SOD of *Fasciola hepatica* (32 kDa) has been reported. The gene has an open reading frame of 438 bp encoding for 146 deduced amino acids.

Comparison of the deduced amino acid sequences of the enzyme to previously reported showed the considerably high homologies. The molecular mass of extracellular Cu/Zn SOD is 60 kDa. N-terminal sequence analysis of cytosolic Cu/Zn SOD of *F. hepatica* showed some similarity to *S. mansoni* cytoplasmic SOD.[68,69] In 2002, the enzyme superoxide dismutase from *Taenia solium* cysticerci (*Ts* SOD) has been reported. It contains 30 kDa molecular weight dimer with 15 kDa monomer. These molecular weights are very similar to those of Cu/Zn SODs from other helminths. The first 25 amino acids in the N-terminal of *Ts* SOD showed 76% similarity to *S. mansoni* Cu/Zn SOD.[70]

In the case of the filarial nematode parasite, the cytosolic and extracellular forms of Cu/Zn SOD from *B. pahangi* have been reported. They are dimeric and their derived amino acid sequences are highly homologous. The *B. pahangi* cytosolic Cu/Zn SOD has 57% identity to its human counterpart whereas the extracellular enzyme is only 35% identical over the cores of the mature proteins. In addition, the parasite extracellular SOD has been shown to have two distinct differences from the extracellular SOD of the human that it has an N-terminal extension truncated by 25 residues and lacks of a C-terminal extension. These features are also found in extracellular SOD of *S. mansoni* and *O. volvulus*.[71] In 1995, Ou X, as coworkers have determined the levels of SOD in different stages of the lymphatic filarial nematode parasite of man, *B. malayi*. They have examined that the highest levels of enzyme activity was found in adult male worm extracts with no significantly difference in the overall levels of SOD in extracts of adult female worms and microfilariae. Recently, it has been reported that the mRNA level of cytosolic Cu/Zn SOD was similar between adults and microfilariae. The extracellular SOD has been determined to express ten times higher in adult worms than in other stages. It has been shown that the adult male worms had higher level of expression than that of the female worms. In addition, the cytosolic SOD have been determined roughly equivalent amounts in males, females, and microfilaria.[72] The intracellular of *O. volvulus* SOD contains 158 amino acids corresponding to molecular mass of 16,339 Da. Homologies analysis showes 56% for the *O. volvulus* and human SOD and 38% for the *O. volvulus* and *S. mansoni*.[73] The SODs of *Trichinella spiralis* and *Dirofilaria immitis* have been partially characterized. The Cu/Zn SOD of *T. spiralis* muscle-stage larva is composed of two identical 17 kDa subunits. Similarly, the Cu/Zn SOD from adult worms of *D. immitis* has a molecular weight of 18 kDa and the active from of enzyme is dimeric.[74,75] The sequences of the parasitic nematode *H. contortus* Cu/Zn

SOD has homology to other helminth Cu/Zn SOD with the highest similarity to *Caenorhabditis elegans*. Two in vitro-expressed proteins in extracts of adult *H. contortus* SOD with molecular masses of approximately 19.8 and 18 kDa have been discovered. An additional protein of approximately 16.8 kDa has been detected in adult ES material.[76]

Effect of inhibitors and enhancers SOD activities have been examined. In 1996, Sanchez and colleagues has demonstrated that Thiabendazole could inhibit the SOD activity of *Ascaris suum*. In 2001, Didion and colleagues has revealed that Diethyldithiocarbamate (DDC) could affect the SOD activity leading to the damage of basilar artery.[77,78]

In 2004, LoVerde and colleagues has reported the vaccination of mice with naked DNA constructs containing Cu/Zn cytosolic SOD (CT-SOD) and showed significant levels to be protective against *S. mansoni* infection. The study also demonstrated that adult worms were subject to immune elimination by vaccination with CT-SOD of *S. mansoni* but not glutathione peroxidase of *S. mansoni*.[79]

Lately, SOD has already been used as the drug target for the treatment of osteoarthritis[80] and cancer. In cancer cell, the Cu/Zn SOD activity is abnormal and can be inhibited by 2-ME (oestrogen derivatives), leading to the accumulation of cellular superoxide ($O^{\cdot}_2$) which can kill the cancer cells.[81,82] It also has been used to diminish the cell damage during tissue reperfusion fllowing oxygen transplant.[52,83]

Since, Cu/Zn SODs have been characterized and purified from various parasites of different species and their sequences features has been shown to be significantly different from those of human. Its possibility of being used as the novel drug target for treatment of diseases is now using *W. bancrofti* as a model. In this study, the gene encoding for Cu/Zn superoxide dismutase from *W. bancrofti* has been isolated, characterized and analyzed in comparison to those of human and other nematodes. The data obtained will be useful for the further studies on drug design for the treatment of nematodes.

Table 1 The *Cu/Zn SOD* gene from various parasites.

Organism	Nucleotide Acession Number	Amino acid Acession Number	Size cDNA (bp)	Molecular weight (Da)	homology to human based on deduced amino acid composition (%)
Human					
extracellular	J02947	AAA66000	723	135,000Da (cps)* [84]	-
intracellular	AY049787	AAL15444	465	32,000Da (cps)* [85]	-
Schistosoma mansoni					
extracellular	M27529	AAA29937	555	20,326[66]	39[66]
intracellular	M97298	AAA29935	462	15,693 [67]	40-45[66]
Onchocerca volvulus					
extracellular	L13778	AAA17049	605	20,904[86]	38.1[86]
intracellular	X57105	CAA40389	477	16,339[73]	56[73]
Driofilaria immitis					
intracellular	AF004949	AAB61472	477	18000[75]	-
Caenorhabditis elegans					
extracellular	AB003924	BAA28262	531	18,100[87]	-
intracellular	L20135	AAA28147	477	-	-
Brugia pahangi					
extracellular	X76283	CAA53901	600	29,000[71]	35-36[71]
intracellular	X76284	CAA53902	477	19,000[71]	55-57[71]
Brugia malayi					
extracellular	-	-	-	28,500+ [72]	-
intracellular	AY428604	AAR06638	477	19,000+ [72]	-

* cps = completed sequences

+ estimate from SDS-PAGE

CHAPTER III

MATERIALS AND METHODS

1. Blood Samples

Microfilariae of *W. bancrofti* infected blood samples were collected from Myanmar workers in the endemic area of Mae Sod district, Tak province, Thailand. Blood samples were previously screened using the traditional Giemsa staining of thick blood smear prior to the parasite isolation using membrane filtration.

2. Isolation of parasites

Five milliliters of microfilaria infected blood was taken from host and transferred to a test tube containing 7 mg/ml of EDTA as an anticoagulant agent. The blood was diluted with equal volume of phosphate buffered saline, pH 7.0 (PBS; 0.137 M NaCl, 10 mM Na_2HPO_4, 3.2mM KH_2PO_4) and filtered through a 5-μm polycarbonate membrane (Minipore). Microfilariae were then resuspended in PBS and centrifuged at 5,000 rpm at 4 °C for 10 min. The pellets of microfilariae were washed with PBS for three times prior to storage at –70 °C until used.

3. Isolation of genomic DNA

The genomic DNA was extracted from filarial parasites using genomic DNA Purification Kit (Gentra). The procedure of isolation was performed according to the instruction manual provided by the company. The microfilaria pellet was digested in 300 μl of Cell Lysis Solution (0.01 M Tris-2%, SDS-0.01 M, EDTA pH 8) and 1.5 μl of Proteinase K Solution (200 mg/ml) at 55 $^{\circ}$C for 4-6 hrs. The lysate was further digested by the addition of 0.12 units of RNase A Solution (1 mg/ml in 5 mM Tris-HCl, pH 8.0) and incubated at 37 °C for 15-60 min. A 100 μl of Protein Precipitation Solution (3 M ammonium acetate pH 7.4) was added to the cell lysate, quickly spinned at high speed for 20 sec and centrifuged at 13,000 rpm for 3 min. Supernatant containing DNA was transferred to a new tube, precipitated by the addition of 300 μl of 100% isopropanol, and centrifuged at 13,000 rpm for 3 min. The supernatant was removed and DNA pellet was washed once with 300 μl of 70% ethanol. The sample was mixed and centrifuged

at 13,000 rpm for 3 min. DNA pellet was dried (air-dry) for 10 min, dissolved in 20 µl of DNA Hydration Solution (TE; 0.01 M Tris-0.001 M, EDTA pH 7.4) and incubated at 65 °C for 60 min. The genomic DNA of parasites was stored at 4 °C until use. DNA concentration was determined using UV spectrophotometer and the absorbance was measured at 260 nm. Molar extinction of DNA was calculated as; 1 O.D. 260 nm = DNA concentration of 50 ng/ml.

4. Primers

The sets of primer were used for amplification of the gene encoding for cytoplasmic Cu/Zn superoxide dismutase from *W. bancrofti* (*Cu/Zn WbSOD*). The primers were designed based on the nucleotide sequence comparison among cytoplasmic Cu/Zn superoxide dismutase genes of *B. pahangi, O. volvulus, D. immitis, S. mansoni* and *H. contortus.*

5. PCR amplification of *Cu/Zn WbSOD* gene

PCR amplifications of partial *Cu/Zn WbSOD* gene of purified parasites DNA were performed using degeneracy primers namely sodF1 and sodR1. (Table 2)

Table 2 Nucleotide sequences of degeneracy primers, sodF1 and sodR1.

Primers	Nucleotide sequences	Tm
Forward primer (sodF1)	5'... GSYGAAATCAARGGTTTAACTCCYGGT... 3'	54 °C
Reverse primer (sodR1)	5'... CAGCRTGWACAACAAKWGAACGMCCMA... 3'	55 °C

Tm = 59.9 + 41[%GC] - [675/Primer Length]

%GC = [#G+#C+#S+E+O+0.5(#N+#X+#M+#Y+#K+#R)+0.6(#B+#V)+ 0.33(#H+#D)]/Total number of all bases

S = G or C Y = C or T

R = G or A W = A or T

K = G or T M = A or C

All reactions were manipulated in 25 µl volume containing 50 ng of genomic DNA in 10x PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 1 µM each of primers, 100 µM each of dATP, dTTP, dCTP and dGTP, 1.5 mM $MgCl_2$ and 1.5 units of *Taq* DNA polymerase (GIBCOBRL®). Sterile distilled water was added to adjust volume to 25 µl. PCR was performed using DNA thermal cycler (MJ Research PTC-200 Peltier thermal cycler) for 30 cycles. Each cycle consisted of denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min and extension at 72 °C for 1 min. The PCR products was analyzed by electrophoresis on 1.5 % agarose gel at 110 Volts for approximately 30 min prior to ethidium bromide staining and observation under ultraviolet light.

6. Cloning of the PCR fragment

6.1 Purification of PCR fragment

The PCR fragment was eluted from the gel and purified using QIAGEN® Purification system prior to cloning. The procedure of purification was performed according to the instruction manual provided by the company. The DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a colorless tube and added 3 volumes of Buffer QG (4 M guanidine thiocyanate pH 7.5) to 1 volumn of gel. Then, the gel slice was incubated at 50 °C for 10 min until it was completely dissolved and its color should be yellow. After that, 100-200 µl of isopropanol was added to the reaction and mixed. This mixture was placed into a QIAquik spin column, centrifuged at 10,000 rpm for 1 min, discarded flow-through and placed QIAquick column back in the same collection tube. Then, the DNA bound QIAquick column was washed with 0.5 ml of Buffer QG and centrifuged at 10,000 rpm for 1 min followed by washing with 0.75 ml of Buffer PE (3 M sodium acetate in 80% ethanol) to QIAquick column and centrifuged at 10,000 rpm for 1 min. The DNA bound QIAquick column was centrifuged again at 10,000 rpm for 1 min and placed QIAquick column into a clean 1.5 ml microcentrifuge tube. The DNA was eluted with 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5). The column was centrifuged at 10,000 rpm for 2 min.

6.2 Ligation of PCR fragment and vector

The purified 200 ng of PCR fragment was further ligated to the pGEM®-T Easy vector using protocol of pGEM® -T Easy Vector Systems (Invitrogen, Promega). The reaction was consisted of 4 µl of PCR product, 1 µl of 2X Rapid Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP 10% PEG), 1 µl of the pGEM®-T Easy vector and 1 µl of T4 DNA Ligase (3 Unit final concentration). The reaction was mixed, and incubated for 12-14 hrs at 4 °C.

6.3 Preparation of ultra-competent *E. coli* cells for transformation

The competent *E. coli* cells were prepared for transformation. Single colony of selected *E. coli* strain (DH5α) was cultured in 5 ml of LB-broth and incubated for 12-14 hrs at 37 °C with moderate agitation (~250 rpm). Then, 1 ml of the overnight culture was added to 150 ml of LB-broth and grown at 37°C with moderate agitation until lag phase. (the OD_{600} nm ≈ 0.5) Subsequently, the culture was placed on ice for 10 min and centrifuged at 5,000 rpm for 10 min at 4°C. The bacteria pellet was washed twice in 10 ml and 5 ml of sterile 10% glycerol and, centrifuged 5,000 rpm at 4°C for 10 min. Finally, the pellet was resuspended in 0.02-0.2 ml of cold 10% glycerol, and aliquots of 40 µl was transferred to a new eppendorf tubes for further transformation or kept at -70°C until use.

6.4 Transformation by electroporation

Electroporation transformation was performed using BioRad MicroPulser (BIO-RAD MicroPulser™) according to the protocols provided by the company. BioRad MicroPulser was set to "Ec 2" for 2 mm cuvettes (2.50kV). Four microliters of the ligation product was added to the competent cells. Transformation reaction was transferred to a chilled electroporation cuvette, and immediately pulsed in the electroporator. Then, 250 µl of LB broth was added to the reaction. The mixture was transferred into 1.5 ml eppendorf tubes and incubated at 37 °C for 45 min. After that, 100 to 150 ul of the mixture was spread on the LB-ampicillin agar plates containing X-gal and IPTG (40 µl of 20 mg/ml X-gal and 10 µl of 200 mg/ml IPTG were spread on the LB-ampicillin agar plates 30 minutes prior to plating the transformations). The plates were incubated for 17 hrs at 37 °C. Colonies containing plasmids without insertion were blue color. Colonies containing plasmids with insertion remained white and were selected for further examination by transferring to a new LB–ampicillin agar plate containing X-gal and IPTG for verification of the phenotype.

7. Recombinant DNA extraction

White colonies containing the recombinant DNAs were selected and cultured in 5 ml of LB media in the presence of 100 μg/ml of ampicilin for 24 hrs. The recombinant DNAs were purified using QIAGEN® QIAprep Spin Miniprep Kit Protocol. The procedure of extraction was performed according to the instruction manual provided by the company. The *E. coli* cells containing recombinant DNA were grown in 5 ml of LB broth for 12-14 hrs at 37 °C. Each cultures was transferred to a microcentrifuge tube and centrifuged for 1 min at 5,000 rpm to harvest the cells using an eppendrof microcentrifuge. The supernatant was removed and each cell pellet was resuspended by spinning in 250 μl of "Buffer P1" (2 mg/ml lysozyme, 50 mM glucose, 10 mM CDTA (cyclohexane diamine tetracetate), 25 mM Tris-HCl, pH 8.0). After resuspension of the pellet, 250 μl of Buffer P2 (0.2 N NaOH, 1% SDS (sodium dodecyl sulfate)) was added to the mixture and the tube was gently inverted for 4-6 times. Then, 350 μl of Buffer N3 (3 M NaOAC (sodium acetate), pH 4.8) was added and the solution was mixed gently but thoroughly, immediately after addition of Buffer N3. All reaction were centrifuged at 13,000 rpm for 10 min. Then, the supernatant was applied from top step into the QIAprep Spin Column, centrifuged at 10,000 rpm for 1 min. After that, QIAprep Spin Column was washed by the addition of 0.5 ml of Buffer PB (contains guanidine hydrochloride and isopropanol) followed by centrifugation at 10,000 rpm for 1 min. Subsequently, QIAprep Spin Column was washed with 0.75 ml of Buffer PE (3 M sodium acetate in 80% ethanol and centrifuged at 10,000 rpm for 1 min. The flow-through was discarded and the the column was placed for centrifugation at 10,000 rpm for 1 min. The QIAprep column was transferred a clean 1.5 ml microcentrifuge tube and DNA was eluted with 20 μl of Buffer EB (10 mM Tris-Cl, pH 8.5) prior to centrifugation at 10,000 rpm for 2 min.

8. Analysis of inserted *Cu/Zn WbSOD* gene in recombinant DNA

The purified recombinant DNAs were tested by PCR amplification and restriction enzyme digestion. The PCR reaction was performed according to the methods described above. Each of restriction enzyme digestions was manipulated in 20 μl volume containing 100-200 ng of the purified recombinant DNA, 10x buffer *Eco*RI (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 0.02% Triton X-100, 0.1 mg/ml BSA.) and 10 unit of *Eco*RI (GIBCOBRL®). The reaction was subsequently incubated at 37 °C for 4 hrs followed by

heating at 68 °C for 10 min. The PCR product or the digested product was analyzed by electrophoresis on 1.2% agarose gel at 110 Volts prior to ethidium bromide staining and observation under ultraviolet light.

9. DNA sequencing of *Cu/Zn WbSOD* gene

The DNA sequence analysis was performed by BSU (Bioservice Unit), National Science and Technology Development Agency (NSTDA) as followed;

DNA sequencing was performed using Big Dye Terminator Cycle Sequencing procedure and analyzed using ABI PRISM 3100 (Perkin Elmer). The PCR condition for DNA sequencing was accomplished by using 25 cycles of denaturation at 95 °C for 5 secs, annealing at 50 °C for 4 sec and extension at 60 °C for 4 min. The nucleotide sequencing data was analyzed by using software of ABI PRISM Model 3100 version 3.7 and analysis procedure was accomplished according to the manual instruction provided from company.

10. Analysis of the partial nucleotide sequences of *Cu/Zn WbSOD*

The partial nucleotide sequences of *Cu/Zn WbSOD* were analyzed using BLAST program from http://www.ncbi.nlm.gov/BLAST. The nucleotide sequence of interested gene fragment was compared to nucleotide sequences of various organisms that have been previously submitted in GenBank.

11. Investigation of the 5' and 3' end of *Cu/Zn WbSOD* gene

Another sets of primers were designed for PCR amplification to complete the *Cu/Zn WbSOD* gene. Two pairs of degenerate primers were designed based on the nucleotide sequence comparison of *Cu/Zn SOD* gene of *B. malayi*, *B. pahangi*, *D. immitis* and *W. bancrofti*. (Figure 7) All reactions of PCR were manipulated in 25 µl volume containing 50 ng of genomic DNA in 10x PCR buffer, 1 µM each of primers, 100 µM of dNTPs, 1.5 mM $MgCl_2$ and 1.5 units of *Taq* DNA polymerase. Sterile distilled water was added to make volume to 25 µl. A PCR was performed for 30 cycles. Each cycle consisted of initial denaturation step at 94 °C for 4 min, denaturation at 94 °C for 1 min, annealing for 1 min, extension at 72 °C for 1 min. The

additional extension step was performed at 72 °C for 10 min. The primers and steps were shown in Table 3 and Figure 8.

12. Complete sequence of *Cu/Zn WbSOD* gene

The nucleotide sequence data was analyzed using the BLAST program of http://www.ncbi.nlm.nih.gov/BLAST. The percentage of nucleotide and amino acid sequence homology in comparison to various organisms were reported. All sequence data were analyzed using Clustal W sequence alignment program for The European Molecular Biology Laboratory (EMBL, U.K.) at http://www.ebi.ac.uk/. The predicted protein molecular weight was analyzed using calculates the molecular weight program at http://bioinformatics.org/sms/index.html. Restriction enzyme mapping and hydrophobicity/ hydrophilicity were analyzed using GENETYX-WIN program (version 5.0). All alignment data were inspected with sequence data in GenBank using PAUP program version 4.0 for construction of evolutionary trees of *Cu/Zn Wb-SOD*.

Table 3 Nucleotide sequences of primers for PCR amplification to complete the *Cu/Zn WbSOD* gene.

Primers	Names	Nucleotide sequences (5' ⟶ 3')	Tm (°C)
Forward primers	WbS	... ATGAGTGCRARTCGRATAGCTGT...	55
	Ater	... CAATACGGTGATACTACAAACGG...	48
Reverse primers	WbE	..... CAAYRATACCACAYGCAACACG....	54
	Zter	..... AACACGRGSACCAGCATTAMC.....	49
	Bmp	... CAATTCAAGAAGCAGCACYAAYG...	48

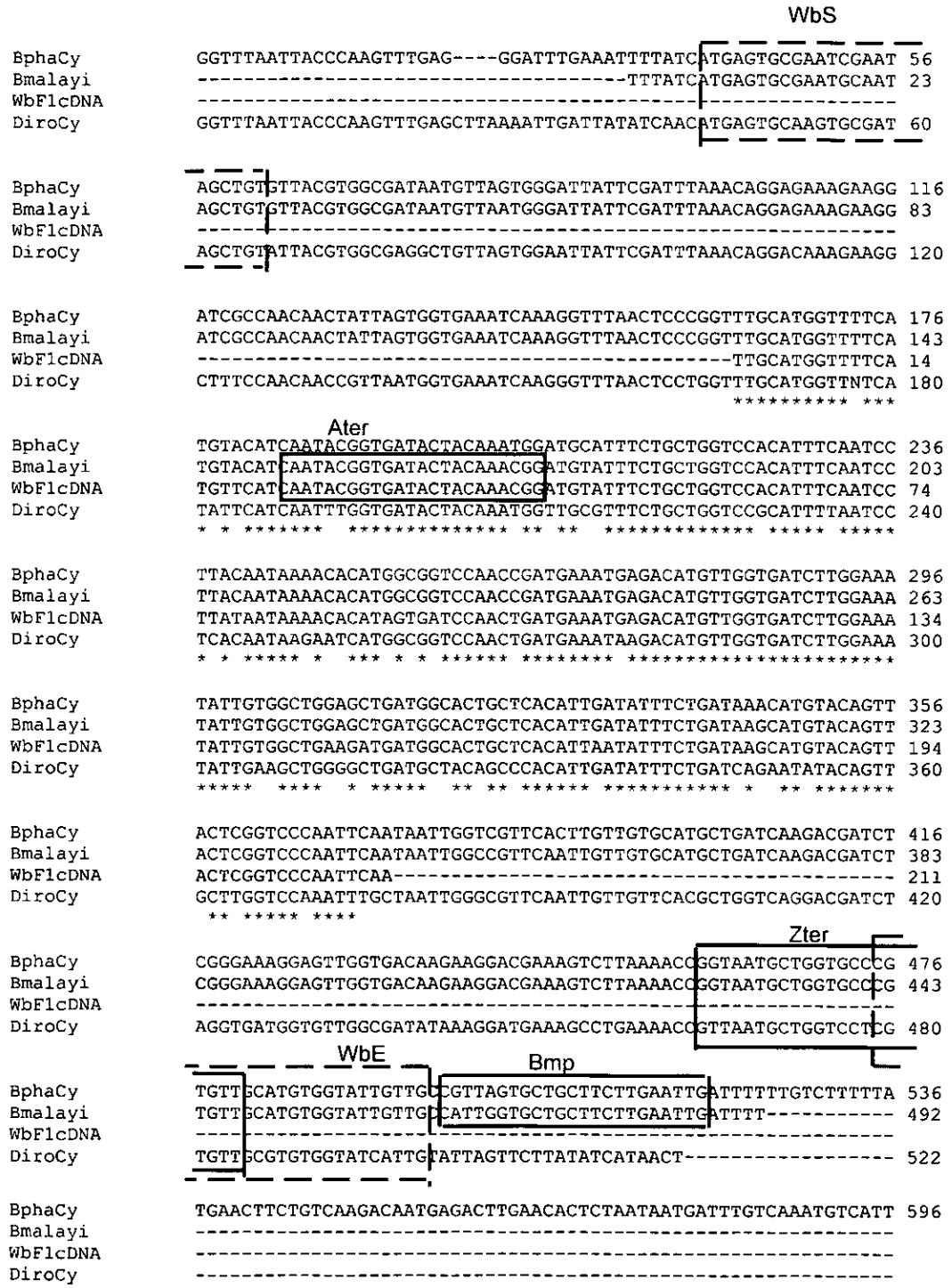


Figure 7 The regions of *Cu/Zn SOD* for primer design (WbS/WbE, Ater/Zter and Bmp). BphaCy, Bmalayi, WbF1cDNA and DiroCy represent cytosol *Cu/Zn SOD* of *B. pahangi*, *B. malayi*, *W. bancrofti* recombinant DNA of Swb1 and *D. immitis*, respectively.

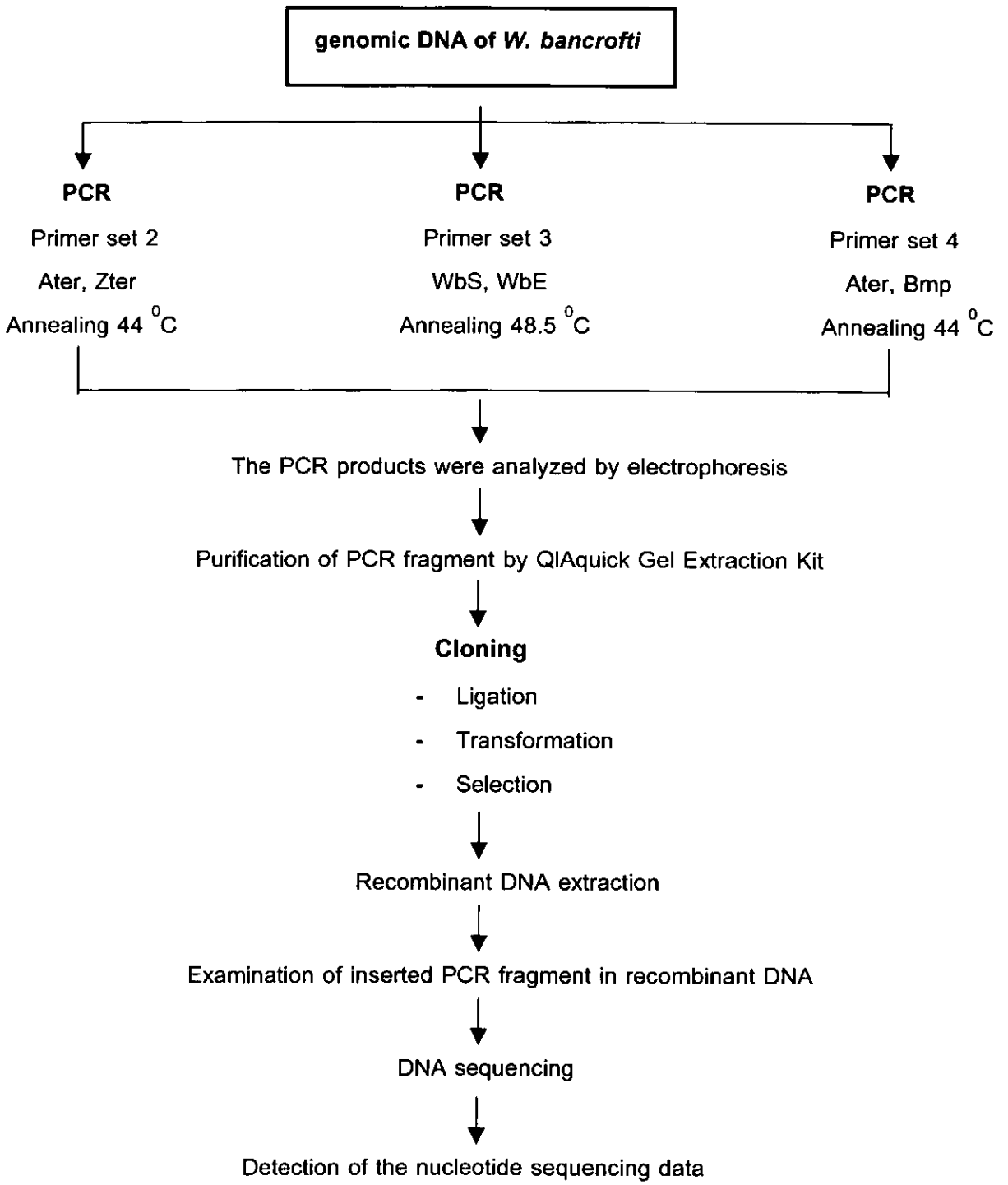


Figure 8 Steps for achievement of complete *Cu/Zn WbSOD* gene.

CHAPTER IV

RESULTS

1. *W. bancrofti* genomic DNA extraction

The genomic DNA of *W. bancrofti* was extracted from one sample as described in Materials and Methods 3. Agarose gel electrophoresis was used to determine genomic DNA before PCR amplification. A single band of high molecular weight DNA sized more than 23 kb was observed after ethidium bromide staining as shown in Figure 9. The quantity and purity of the DNA were investigated by measurement of the absorbance at 260 nm and 280 nm and showed that they were 0.001 and 0.002 with the ratio of A260/A280 = 0.5.

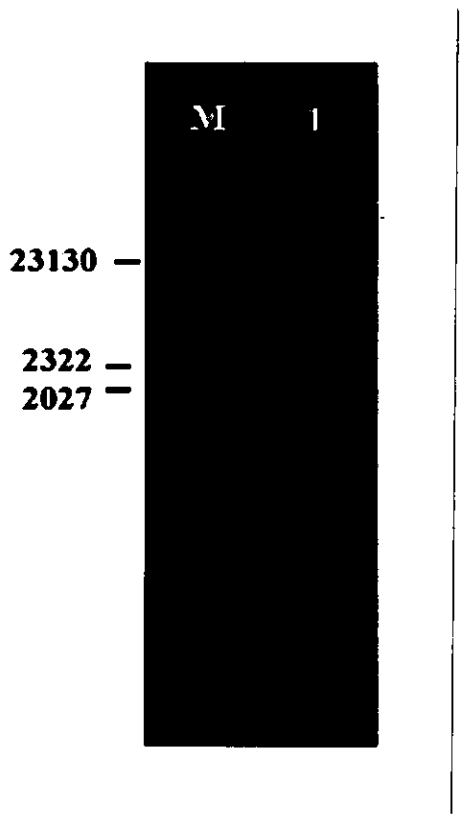


Figure 9 Electrophoresis profile of 50 ng *W. bancrofti* genomic DNA in 0.8% agarose gel.

Lane M: Lamda *Hind* III marker

Lane 1: 50 ng of *W. bancrofti* genomic DNA

3. Nucleotide sequences of partial *Cu/Zn WbSOD* gene

PCR product of 600 bp in size was used for cloning and DNA sequencing (Materials and Methods 6-9). Recombinant DNA of Swb1 clone were selected and DNA sequence data was shown in Figure 11.

```
CLUSTAL W multiple sequence alignment

sodF1           TTGCATGGTTTTC-TGTTCATCAATACGGTGATACTACAAACGGATGTATTTCTGCTGGT 59
sodR            --GCATGGTTTTCATGTTCATCAATACGGTGATACTACAAACGGATGTATTTCTGNTGGT 58
                  *********** ****************************************** ****

sodF1           CCACATTTCAATCCTTATAATAAAACACATAGTGATCCAACTGTTAGTTTTATTCTATCT 119
sodR            CCACATTTCAATCCTTATAATAAAACACATAGTGATCCAACTGTTAGTTTTATTCTATCT 118
                ************************************************************

sodF1           CCGAGGAAAAAAGGAATTACATTAAATATATGTTAGTTTTATTGGTTTAATTTCAGGAAC 179
sodR            CCGAGGAAAAAAGGAATTACATTAAATATATGTTAGTTTTATTGGTTTAATTTCAGGAAC 178
                ************************************************************

sodF1           AATACCTGGTTCTAAATGTTTATGAAATATGACGTAGATCGAAAAAAAATTTTTTTTTTT 239
sodR            AATACCTGGTTCTAAATGTTTATGAAATATGACGTAGATCGAAAAAAAATCTTTTTTTTT 238
                ************************************************* *********

sodF1           AATTTTAATATGAGATTGATAATTCTTTTATCTTTACTCGTAACTCACAGGTGATCTTTA 299
sodR            AATTTTAATATGAGATTGATAATTCTTTTATCTTTACTCGTAACTCACAGGTGATCTTTA 298
                ************************************************************

sodF1           GGATGAAATGAGACATGTTGGTGATCTTGGAAATATTGTGGCTGAAGATGATGGCACTGC 359
sodR            GGATGAAATGAGACATGTTGGTGATCTTGGAAATATTGTAGCTGAAGGTGATGGCACTGC 358
                ************************************ ******* ************

sodF1           TCACATTAATATTTCTGATAAGCATGTACAGGTATAACAAAATTACTTCAGACTTTTTTA 419
sodR            TCACATTAATATTTCTGATAAGCATGTACAGGTATAACAAAATTACTTCAGACTTTTTTA 418
                ************************************************************

sodF1           TATTTCTCGACATCTAATCAAATTTTATACTTATATTTTCTTATTTCCAGTTACTCGGTC 479
sodR            TATTTCTCGACATCTAATCAAATTTTATACTTATATTTTCTTATTTCCAGTTACTCGGTC 478
                ************************************************************

sodF1           CCAATTCAA- 488
sodR            CCAATTCAAA 488
                *********
```

Figure 11 Forward (sodF1) and reverse (sodR) nucleotide sequence data of *Cu/Zn WbSOD* from Swb1 clone.

BLAST program was used for identification of *Cu/Zn WbSOD* gene in comparison to the previously reported data from GenBank. The nucleotide sequence comparison that revealed *Cu/Zn WbSOD* was similar to those of *B. malayi* (*Cu/Zn BmSOD*) (Figure 12).

```
CLUSTAL W (1.82) multiple sequence alignment

sodF1;Cu/ZnWbSOD(DNA) TTGCATGGTTTTCATGTTCATCAATACGGTGATACTACAAACGGATGTATTTCTGCTGGT 60
Cu/ZnBmSOD(cDNA)      ------------------------------------------------------ATGAGT 6
                                                                               **

sodF1;Cu/ZnWbSOD(DNA) CCACATTTCAATCCTTATAATAAAACACATAGTGATCCAACTGTTAGTTTTATTCTATCT 120
Cu/ZnBmSOD(cDNA)      GCGAATG-CAATAGCTGTGTTA-----CGTGGCGATAATGTTAATGGGATTATTCGATTT 60
                       *  **  ****   * *  **     * * * ***     *  * *  ****** ** *

sodF1;Cu/ZnWbSOD(DNA) ---CCGAGGAAAAAAGGAATTACATTAAATAT--ATGTTAGTTTTATTGGTTTAATTTCA 175
Cu/ZnBmSOD(cDNA)      AAACAGGAGAAAGAAGGATCGCCAACAACTATTAGTGGTGAAATCAAAGGTTTAACTCCC 120
                         * *  **** *****    **  ** ***   ** *    * *  ******* * *

sodF1;Cu/ZnWbSOD(DNA) GGAACAATACCTGGTTCTAAATGTTTATGAAATATGACGTAGATCGAAAAAAAATTTTTT 235
Cu/ZnBmSOD(cDNA)      GGTT---TGCATGGTTTTCA-TGTACATCAA-TACGGTG-ATACTACAAACGGATGTATT 174
                      **     * * ***** * * ***  ** ** ** *  * * *    ***   ** * **

sodF1;Cu/ZnWbSOD(DNA) TTTTTAATTTTAATATGAGATTGATAATTCTTTTATCTTTACTCGTAACTCACA-GGTGA 294
Cu/ZnBmSOD(cDNA)      TCT---------------GCTGGTCCACATTTCAATCCTTACA-ATAAAACACATGGCGG 218
                      * *               * * *   *   **  *** ****   ***  **** ** *

sodF1;Cu/ZnWbSOD(DNA) TCTTTAGGATGAAATGAGACATGTTGGTGATCTTGGAAATATTGTGGCTGAAGATGATGG 354
Cu/ZnBmSOD(cDNA)      TCCAACCGATGAAATGAGACATGTTGGTGATCTTGGAAATATTGTGGCTGGAGCTGATGG 278
                      **     ****************************************** ** ******

sodF1;Cu/ZnWbSOD(DNA) CACTGCTCACATTAATATTTCTGATAAGCATGTACAGGTATAACAAAATTACTTCAGACT 414
Cu/ZnBmSOD(cDNA)      CACTGCTCACATTGATATTTCTGATAAGCATGTACAGTTACTCGGTCCCAATTCAATAAT 338
                      ************* ********************** **          * *  * * *

sodF1;Cu/ZnWbSOD(DNA) TTTTTAT---ATTTCTCGACAT-CTAATCAA----ATTTTATACTTATATTTTCTTATTT 466
Cu/ZnBmSOD(cDNA)      TGGCCGTTCAATTGTTGTGCATGCTGATCAAGACGATCTCGGGAAAGGAGTTGGTGACAA 398
                      *     *   ***  *   *** ** *****    ** *          * **  * *

sodF1;Cu/ZnWbSOD(DNA) CCAGTTACTCGGTCCCAATTCAA----------------------------------- 489
Cu/ZnBmSOD(cDNA)      GAAGGACGAAAGTCTTAAAACCGGTAATGCTGGTGCCCGTGTTGCATGTGGTATTGTTGC 458
                        **       ***  **  *

sodF1;Cu/ZnWbSOD(DNA) -------------------
Cu/ZnBmSOD(cDNA)      CATTGGTGCTGCTTCTTGA 477
```

Figure 12 Nucleotide sequence comparison of partial *Cu/Zn WbSOD* gene against *Cu/Zn BmSOD* gene (AY428604) from GenBank using BLAST program.

The coding sequences of *Cu/Zn WbSOD* was analyzed using translate program of http://www.expasy.org/tools/dna.html in comparison to *Cu/Zn BmSOD*. (Figure 13-14)

```
CLUSTAL W multiple sequence alignment

sodFl;Cu/Zn Wb-SOD          ------------------------------------------------------------
Cu/Zn Bm-SOD                ATGAGTGCGAATGCAATAGCTGTGTTACGTGGCGATAATGTTAATGGGATTATTCGATTT 60


sodFl;Cu/Zn Wb-SOD          ------------------------------------------------------------
Cu/Zn Bm-SOD                AAACAGGAGAAAGAAGGATCGCCAACAACTATTAGTGGTGAAATCAAAGGTTTAACTCCC 120


sodFl;Cu/Zn Wb-SOD          ---TTGCATGGTTTTCATGTTCATCAATACGGTGATACTACAAACGGATGTATTTCTGCT 57
Cu/Zn Bm-SOD                GGTTTGCATGGTTTTCATGTACATCAATACGGTGATACTACAAACGGATGTATTTCTGCT 180
                               ********** ********** ***************************************


sodFl;Cu/Zn Wb-SOD          GGTCCACATTTCAATCCTTATAATAAAACACATAGTGATCCAACTGATGAAATGAGACAT 117
Cu/Zn Bm-SOD                GGTCCACATTTCAATCCTTACAATAAAACACATGGCGGTCCAACCGATGAAATGAGACAT 240
                            ********** ********** * * ****** **********


sodFl;Cu/Zn Wb-SOD          GTTGGTGATCTTGGAAATATTGTGGCTGAAGATGATGGCACTGCTCACATTAATATTTCT 177
Cu/Zn Bm-SOD                GTTGGTGATCTTGGAAATATTGTGGCTGGAGCTGATGGCACTGCTCACATTGATATTTCT 300
                            ********** ** ********** ********


sodFl;Cu/Zn Wb-SOD          GATAAGCATGTACAGTTACTCGGTCCCAATTCAA---------- 211
Cu/Zn Bm-SOD                GATAAGCATGTACAGTTACTCGGTCCCAATTCAATAATTGGCCGTTCAATTGTTGTGCAT 360
                            **********


sodFl;Cu/Zn Wb-SOD          ------------------------------------------------------------
Cu/Zn Bm-SOD                GCTGATCAAGACGATCTCGGGAAAGGAGTTGGTGACAAGAAGGACGAAAGTCTTAAAACC 420


sodFl;Cu/Zn Wb-SOD          ------------------------------------------------------------
Cu/Zn Bm-SOD                GGTAATGCTGGTGCCCGTGTTGCATGTGGTATTGTTGCCATTGGTGCTGCTTCTTGAATT 480


sodFl;Cu/Zn Wb-SOD          ------
Cu/Zn Bm-SOD                GATTTT 486
```

Figure 13 Nucleotide sequence comparison of *Cu/Zn BmSOD* (cDNA) (AY428604) to partial sequences of *Cu/Zn WbSOD* (cds= coding sequences) using translate program of http://www.expasy.org/tools/dna.html.

```
CLUSTAL W (1.82) multiple sequence alignment

Cu/Zn Wb-SOD    ----------------------------------------LHGFHVHQYGDTTNGCISA 19
Cu/Zn Bm-SOD    MSANAIAVLRGDNVNGIIRFKQEKEGSPTTISGEIKGLTPGLHGFHVHQYGDTTNGCISA 60
                                                        ********************

Cu/Zn Wb-SOD    GPHFNPYNKTHSDPTDEMRHVGDLGNIVAEDDGTAHINISDKHVQLLGPNS--------- 70
Cu/Zn Bm-SOD    GPHFNPYNKTHGGPTDEMRHVGDLGNIVAGADGTAHIDISDKHVQLLGPNSIIGRSIVVH 120
                ***********..**************  ******:*************

Cu/Zn Wb-SOD    -------------------------------------
Cu/Zn Bm-SOD    ADQDDLGKGVGDKKDESLKTGNAGARVACGIVAIGAAS 158
```

Figure 14 Deduced amino acid sequence comparison of Cu/Zn BmSOD (AY428604) partial sequences Cu/Zn WbSOD enzyme.

4. Investigation of the 5' and 3' end of *Cu/Zn WbSOD* gene

The set of primers were designed for PCR to complete the *Cu/Zn WbSOD* gene. *W. bancrofti* DNA were amplified using Ater/Zter, WbS/WbE and Ater/Bmp at the annealing temperatures of 44 °C, 48.5 °C and 44 °C, respectively. Figures 15-17 showed that the PCR product of 600, 1000 and 600 bp in size were obtained, respectively.

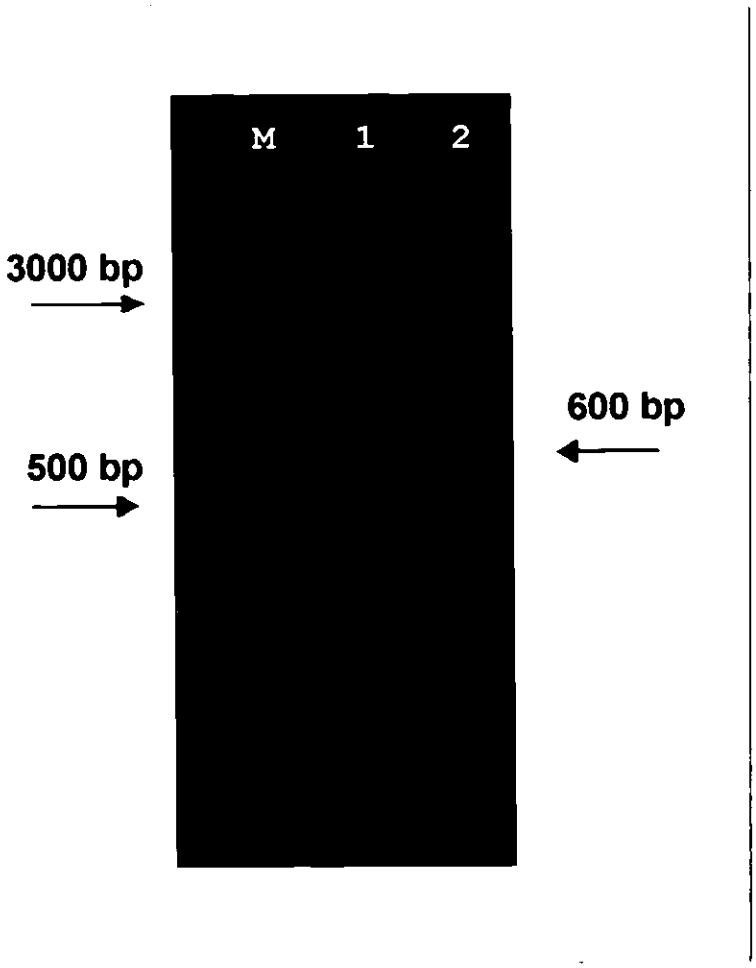


Figure 15 Electrophoresis profile on 1.2% agarose gel of 600 bp PCR product using Ater/Zter as primers.

Lane M = 100 bp DNA ladder marker

Lane 1-2 = PCR product

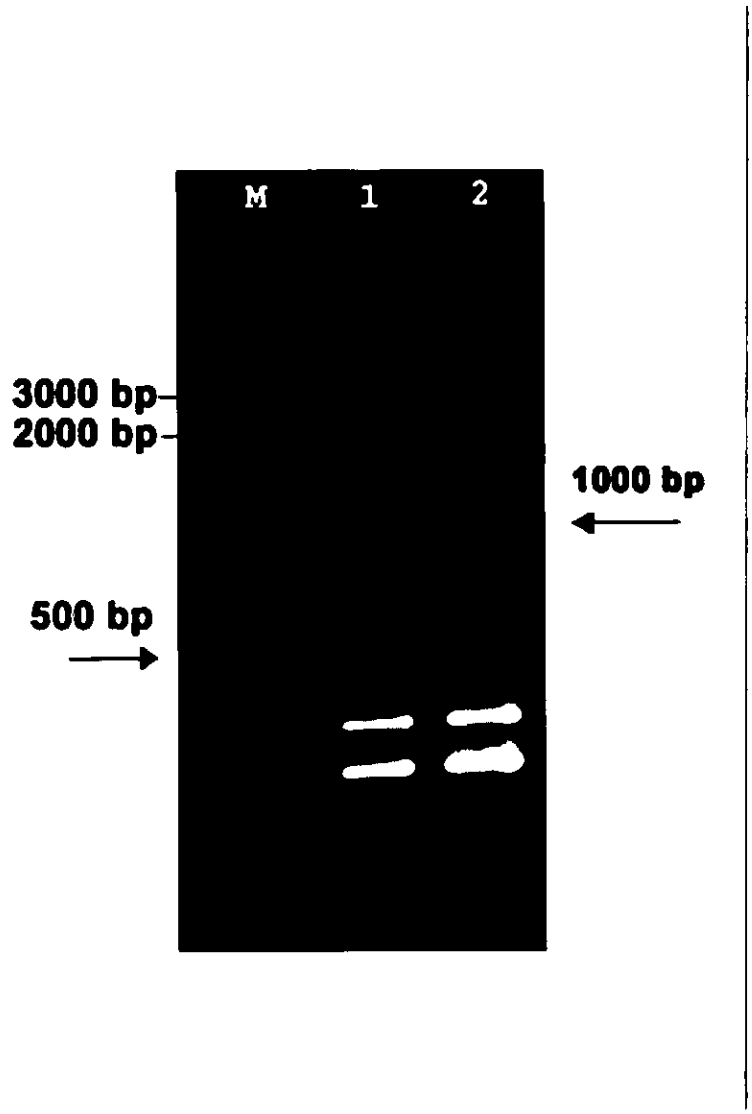


Figure 16 Electrophoresis profile on 1.5% agarose gel of 1000 bp PCR product using WbS/WbE as primers.

Lane M = 100 bp DNA ladder marker

Lane 1-2 = PCR product using WbS/WbE as primers

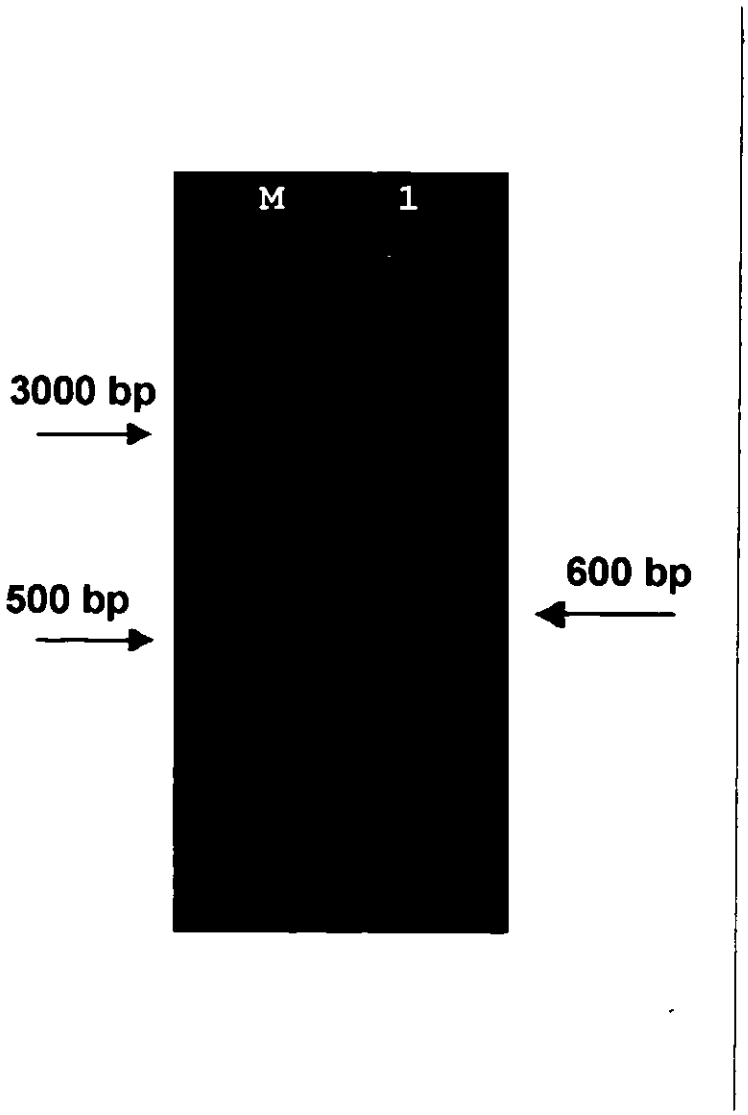


Figure 17 Eletrophoresis profile of 700 bp PCR product using Ater/Bmp as primers on 1.2% agarose gel.

Lane M = 100 bp marker

Lane 1 = PCR product from PCR amplification using Ater/Bmp as primer

5. DNA sequencing of *Cu/Zn WbSOD* completed gene

5.1 Nucleotide sequence analysis

PCR products of *Cu/Zn WbSOD* gene from PCR amplification using Ater/Zter, WbS/WbE and Ater/Bmp were used for cloning and DNA sequencing. The data revealed that *Cu/Zn WbSOD* gene consisted of 1,037 nucleotides containing 4 exon and 3 intron regions. (Figure 18) The predicted coding region (477 nucleotides) was corresponded to 158 amino acids with the calculated molecular mass of 16.34 kDa. Nucleotide sequences and deduced amino acids were shown in Figure 19.

Exon 1
ATGAGTGCAAATGCAATAGCTGTATTACGTGGTGATAATGTTAGTGGGATTATTCGATTTAAACAGGTAA
ATTAAAAAAGGAATTGTAAATAAAACTGTTGGCTTTCTATTAAGCCAACATTTGAATTATTTATTACAAAAAT
TACACATTTTTATTCCTTTGTAATGTAAGTCTCAATAATATTGTTTACACTAGAATAAAGAAAAATAAGTCG
GTATAGTGTAACTTATGGATGTACTTATTCTATTTTCTCTATAGTGCTGTGACACTGCTGTTGAATTGTGGA
TTTTATTATGCAGTAAACATTAACCGTTAAATCAGCGTTTTTAAAGGAAAAAGAAGGATTACCAACAACTA
Exon 2
TTAGTGGTGAAATCAAAGGTTTAACTCCCGGTTTGCATGGTTTTCATGTTCATCAATACGGTGATACTACA
AACGGATGCATTTCTGCTGGTCCACATTTCAATCCTTACAATAAAACACATGGCGGTCCAACCGTTAGTT
TTATTCTATCTCCAAGGGAAAAAGGAATTACGTTAAATATATGTTAGTTTTATCGGTTTAATTTCAGGAACA
ATACCTGGTTCTAAATGTTTATGAAATATGACGTAGATCGAAAAATTTTTTTTTTTTAATTTTAATATGAGATT
Exon 3
GATAATTCTTTTCGTAATTCACAGGTGATCTTTAGGATGAAATGAGACATGTTGGTGATCTTGGAAATATT
GTAGCTGAAGGTGATGGCACTGCTCACATTAATATTTCTGATAAGCATGTACAGGTATAACAAAATTACTT
CAGACTGTTACGTATTTCTCGACATCTAATCAAATTTTATATTTCCGCTTTAATTCTTATATTTTCTTATTCTC
Exon 4
TCATTTCCAGTTACTCGGTCCCAATTCAATAATTGGCCGTTCAATTGTTGTGCATGCTGATCAGGACGAT
CTCGGGAAAGGAGTTGGTGATAAGAAGGACGAAAGTCTTAAAACCGGTAATGCTGGTGCTCGTGTTGC
ATGTGGTATTGTTGCCGTTAGTGCTGCTTCTTGAATTGA

Figure 18 Proposed the *Cu/Zn WbSOD* completed gene consisted of 1,037 nucleotides containing predicted 4 exon and 3 intron regions.

```
M  S  A  N  A  I  A  V  L  R  G  D  N  V  S  G  I  I  R  F  K  Q      22
ATGAGTGCAAATGCAATAGCTGTATTACGTGGTGATAATGTTAGTGGGATTATTCGATTTAAACAG    66

       GTAAATTAAAAAAGGAATTGTAAATAAAACTGTTGGCTTTCTATTAAGCCAACATTTGAA
       TTATTTATTACAAAAATTACACATTTTTATTCCTTTGTAATGTAAGTCTCAATAATATTG
       TTTACACTAGAATAAAGAAAAATAAGTCGGTATAGTGTAACTTATGGATGTACTTATTCT
       ATTTTCTCTATAGTGCTGTGACACTGCTGTTGAATTGTGGATTTTATTATGCAGTAAACA

                                  E  K  E  G  L  P  T  T  I  S  G      33
       TTAACCGTTAAATCAGCGTTTTTAAAG   GAAAAAGAAGGATTACCAACAACTATTAGTGGT    99

E  I  K  G  L  T  P  G  L  H  G  F  H  V  H  Q  Y  G  D  T  T  N      55
GAAATCAAAGGTTTAACTCCCGGTTTGCATGGTTTTCATGTTCATCAATACGGTGATACTACAAAC    165

G  C  I  S  A  G  P  H  F  N  P  Y  N  K  T  H  G  G  P  T            75
GGATGCATTTCTGCTGGTCCACATTTCAATCCTTACAATAAAACACATGGCGGTCCAACC          225

       GTTAGTTTTATTCTATCTCCAAGGGAAAAAGGAATTACGTTAAATATATGTTAGTTTTAT
       CGGTTTAATTTCAGGAACAATACCTGGTTCTAAATGTTTATGAAATATGACGTAGATCGA
       AAAATTTTTTTTTTTAATTTTAATATGAGATTGATAATTCTTTTCGTAATTCACAGGTGA

                D  E  M  R  H  V  G  D  L  G  N  I  V  A  E  G         91
       TCTTTAG  GATGAAATGAGACATGTTGGTGATCTTGGAAATATTGTAGCTGAAGGT       273

D  G  T  A  H  I  N  I  S  D  K  H  V  Q                             105
GATGGCACTGCTCACATTAATATTTCTGATAAGCATGTACAG   GTATAACAAAATTACTT        316

       CAGACTGTTACGTATTTCTCGACATCTAATCAAATTTTATATTTCCGCTTTAATTCTTATA

                                  L  L  G  P  N  S  I  I  G  R  S  I   117
       TTTTCTTATTCTCTCATTTCCAG   TTACTCGGTCCCAATTCAATAATTGGCCGTTCAATT  336

V  V  H  A  D  Q  D  D  L  G  K  G  V  G  D  K  K  D  E  S  L  K    139
GTTGTGCATGCTGATCAGGACGATCTCGGGAAAGGAGTTGGTGATAAGAAGGACGAAAGTCTTAAA  417

T  G  N  A  G  A  R  V  A  C  G  I  V  A  V  S  A  A  S  *          158
ACCGGTAATGCTGGTGCTCGTGTTGCATGTGGTATTGTTGCCGTTAGTGCTGCTTCTTGA        480
```

Figure 19 Proposed nucleotide and deduced amino acid sequences of the *Cu/Zn WbSOD* complete gene.

The amino acid composition of the *Cu/Zn WbSOD* gene revealed that the Cu/Zn WbSOD protein apparently contained 58 neutral (Ala, Asn, Cys, Gln, Gly, Pro), 36 hydrophobic (Ile, Leu, Met, Val), 5 aromatic (Phe, Trp, Tyr), 17 acidic (Asp, Glu), 23 basic (Arg, His, Lys) and 19 hydroxyl-containing amino acid residues (Ser, Thr).

When deduced amino acid sequences of *Cu/Zn WbSOD* gene was compared to those from other organisms, the metal binding site were conserved.[80] The copper-zinc- binding sites containing six histidine (H) and one aspartate (D) residues (His-46, His-48, His-63, His-71, His-80, His-120 and Asp-83)[80,88-89] were indicated in Figure 20. The two cysteines (C) residues (Cys-57 and Cys-149) which form an intrasubunit disulfide bridge in the Cu/Zn SODs were also conserved. The arginine (R) residue (Arg-146) that played important role in guiding the superoxide to the activity site was also presented.[80,88]

Upon homology analysis of the nucleotide sequences of *Cu/Zn WbSOD* gene against the intracellular *Cu/Zn SOD* from *C. elegans*, *O. volvulus*, *D. immitis*, *B. pahangi*, *B. malayi* and human, the 63, 86, 83, 95, 96 and 48% similarity were obtained, respectively. When *Cu/Zn WbSOD* complete coding sequences were compared to the extracellular *Cu/Zn SOD* of *C. elegans*, *O. volvulus*, *B. pahangi* and human, the similarity of 43, 72, 89 and 7% were observed, respectively. The similarity upon comparison of deduced amino acid sequences of *Cu/Zn WbSOD* gene against those of other intracellular Cu/Zn SOD from *C. elegans*, *O. volvulus*, *D. immitis*, *B. pahangi*, *B. malayi* and human were 66, 87, 77, 96, 95 and 56%, respectively. Amino acid sequence comparison of Cu/Zn WbSOD to those of the extracellular Cu/Zn SOD of *C. elegans*, *O. volvulus*, *B. pahangi* and human showed 39, 61, 81 and 30%, respectively. (Figure 20-21 and Table 4)

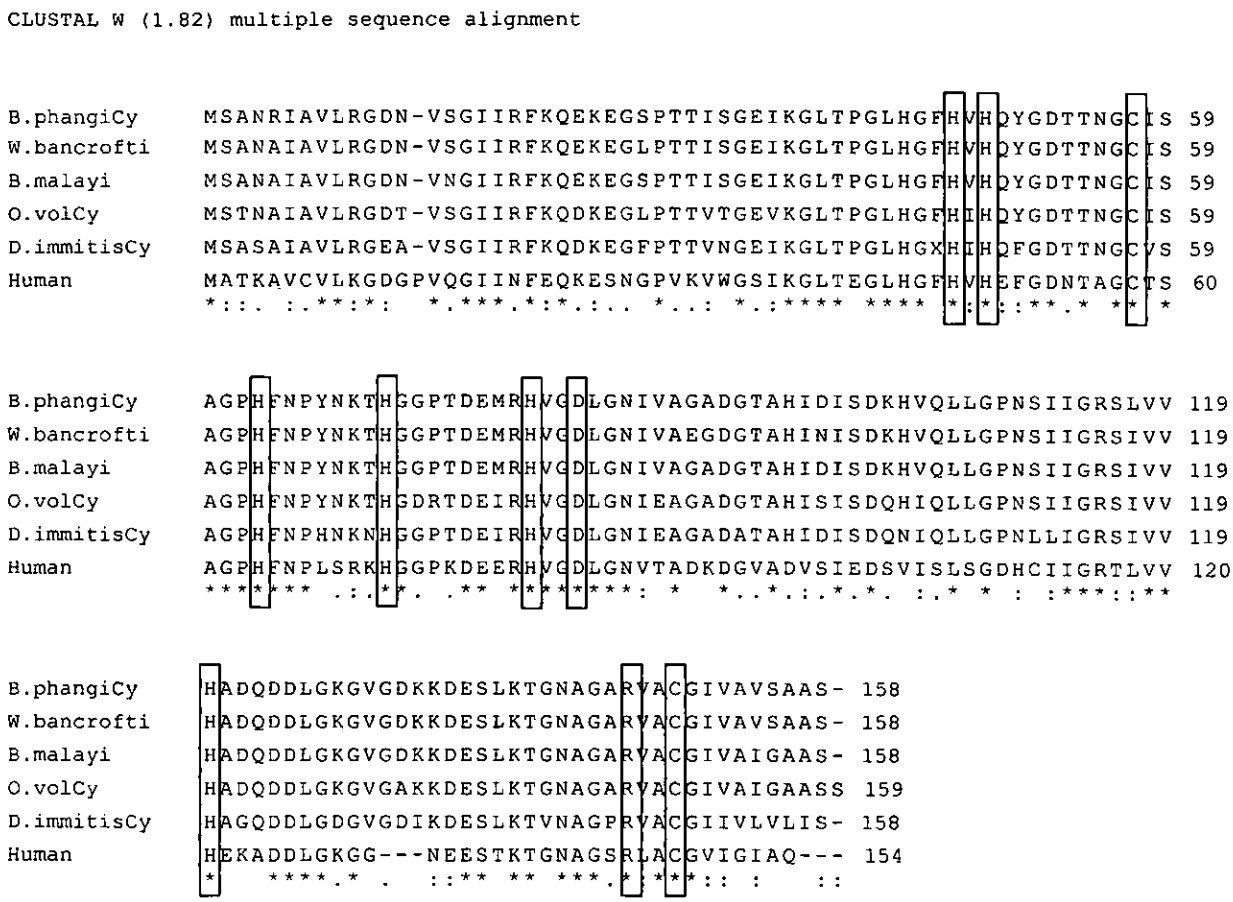


Figure 20 Amino acid sequence alignment of Cu/Zn Wb/SOD compared to intracellular Cu/Zn SOD of *B. pahangi, B. malayi, O. volvulus, D. immitis* and Human. The conserved amino acids required for enzymes activity were indicated in boxes as followed; copper-zinc binding residues (His-46, His-48, His-63, His-71, His-80, His-120 and Asp-83), disulfide bond residues (Cys-57 and Cys-149) and superoxide guiding-to-active-site residue (Arg-146).

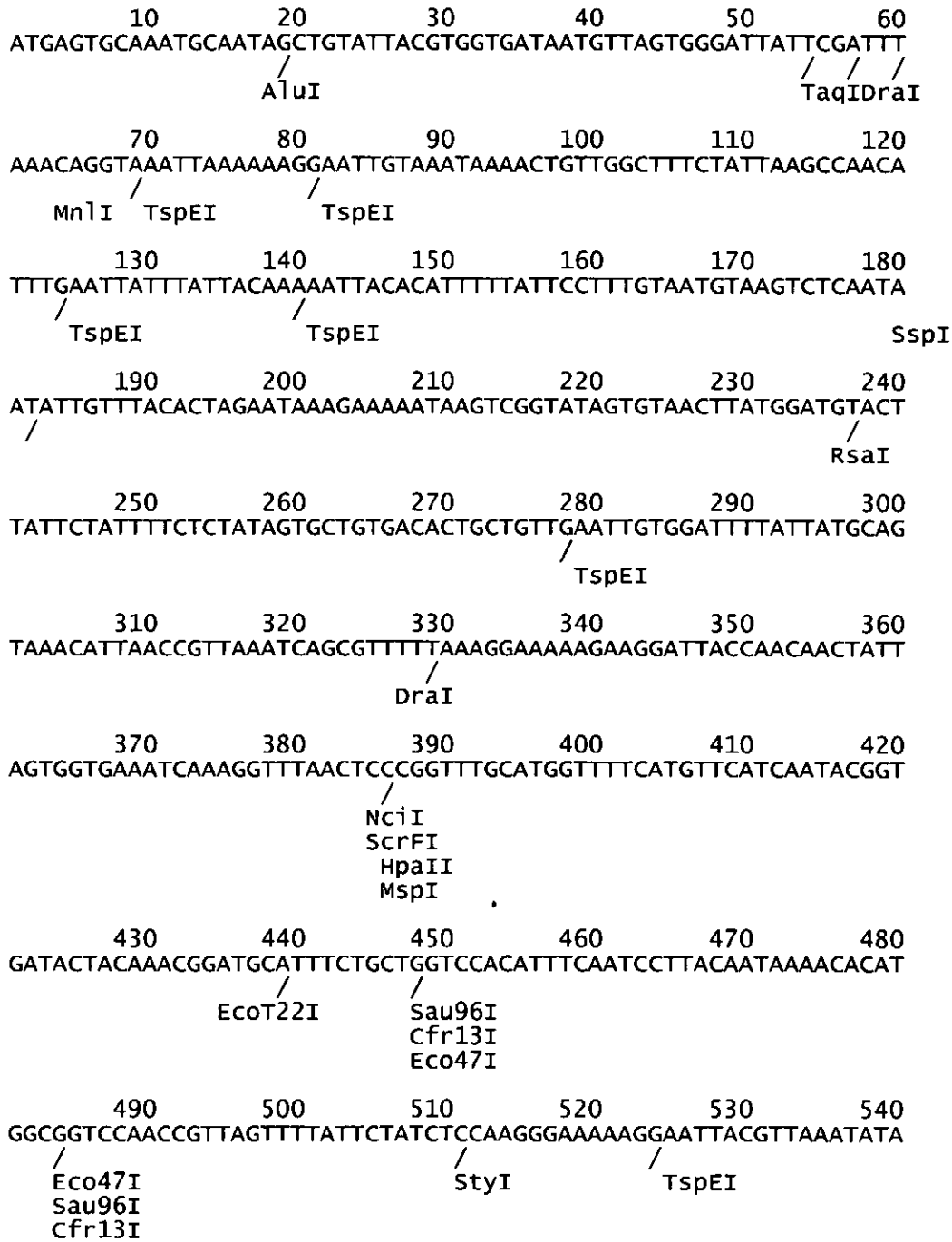
```
CLUSTAL W (1.82) multiple sequence alignment

B.phangi        ATGAGTGCGAATCGAATAGCTGTGTTACGTGGCGATAATGTTAGTGGGATTATTCGATTT 60
B.malayi        ATGAGTGCGAATGCAATAGCTGTGTTACGTGGCGATAATGTTAATGGGATTATTCGATTT 60
W.bancrofti     ATGAGTGCAAATGCAATAGCTGTATTACGTGGTGATAATGTTAGTGGGATTATTCGATTT 60
O.volvulus      ATGAGTACAAACGCGATAGCAGTATTGCGTGGCGATACTGTTAGTGGAATTATTCGATTT 60
D.immitis       ATGAGTGCAAGTGCGATAGCTGTATTACGTGGCGAGGCTGTTAGTGGAATTATTCGATTT 60
                ****** * *     ***** ** ** ***** **   ***** *** ***********

B.phangi        AAACAGGAGAAAGAAGGATCGCCAACAACTATTAGTGGTGAAATCAAAGGTTTAACTCCC 120
B.malayi        AAACAGGAGAAAGAAGGATCGCCAACAACTATTAGTGGTGAAATCAAAGGTTTAACTCCC 120
W.bancrofti     AAACAGGAAAAAGAAGGATTACCAACAACTATTAGTGGTGAAATCAAAGGTTTAACTCCC 120
O.volvulus      AAACAGGACAAGGAAGGCTTACCAACAACCGTTACTGGTGAAGTCAAAGGTTTGACTCCT 120
D.immitis       AAACAGGACAAAGAAGGCTTTCCAACAACCGTTAATGGTGAAATCAAGGGTTTAACTCCT 120
                ******** ** ***** *  ********  *** ******* **** ***** *****

B.phangi        GGTTTGCATGGTTTTCATGTACATCAATACGGTGATACTACAAATGGATGCATTTCTGCT 180
B.malayi        GGTTTGCATGGTTTTCATGTACATCAATACGGTGATACTACAAACGGATGTATTTCTGCT 180
W.bancrofti     GGTTTGCATGGTTTTCATGTTCATCAATACGGTGATACTACAAACGGATGCATTTCTGCT 180
O.volvulus      GGTTTACATGGTTTTCATATTCATCAGTATGGTGACACGACAAATGGGTGCATTTCTGCG 180
D.immitis       GGTTTGCATGGTTNTCATATTCATCAATTTGGTGATACTACAAATGGTTGCGTTTCTGCT 180
                ***** ******* **** * ***** *  ***** ** ***** ** **  *******

B.phangi        GGTCCACATTTCAATCCTTACAATAAAACACATGGCGGTCCAACCGATGAAATGAGACAT 240
B.malayi        GGTCCACATTTCAATCCTTACAATAAAACACATGGCGGTCCAACCGATGAAATGAGACAT 240
W.bancrofti     GGTCCACATTTCAATCCTTACAATAAAACACATGGCGGTCCAACCGATGAAATGAGACAT 240
O.volvulus      GGTCCGCATTTCAATCCTTACAATAAAACCCATGGCGATCGAACTGATGAAATAAGACAT 240
D.immitis       GGTCCGCATTTTAATCCTCACAATAAGAATCATGGCGGTCCAACTGATGAAATAAGACAT 240
                ***** ***** ****** ******* *  ******* ** *** ******** ******

B.phangi        GTTGGTGATCTTGGAAATATTGTGGCTGGAGCTGATGGCACTGCTCACATTGATATTTCT 300
B.malayi        GTTGGTGATCTTGGAAATATTGTGGCTGGAGCTGATGGCACTGCTCACATTGATATTTCT 300
W.bancrofti     GTTGGTGATCTTGGAAATATTGTAGCTGAAGGTGATGGCACTGCTCACATTAATATTTCT 300
O.volvulus      GTTGGTGATCTTGGAAATATTGAAGCTGGAGCCGATGGTACTGCTCACATTAGCATTTCC 300
D.immitis       GTTGGTGATCTTGGAAATATTGAAGCTGGGGCTGATGCTACAGCCCACATTGATATTTCT 300
                ***********************  ****  *  ****  ** ** ******   *****

B.phangi        GATAAACATGTACAGTTACTCGGTCCCAATTCAATAATTGGTCGTTCACTTGTTGTGCAT 360
B.malayi        GATAAGCATGTACAGTTACTCGGTCCCAATTCAATAATTGGCCGTTCAATTGTTGTGCAT 360
W.bancrofti     GATAAGCATGTACAGTTACTCGGTCCCAATTCAATAATTGGCCGTTCAATTGTTGTGCAT 360
O.volvulus      GATCAACATATACAGTTGCTGGGTCCGAATTCGATAATTGGCCGTTCAATTGTTGTACAT 360
D.immitis       GATCAGAATATACAGTTGCTTGGTCCAAATTTGCTAATTGGGCGTTCAATTGTTGTTCAC 360
                *** *  ** ******* ** ***** ****   ******* ****** ******* **

B.phangi        GCTGATCAAGACGATCTCGGGAAAGGAGTTGGTGACAAGAAGGACGAAAGTCTTAAAACC 420
B.malayi        GCTGATCAAGACGATCTCGGGAAAGGAGTTGGTGACAAGAAGGACGAAAGTCTTAAAACC 420
W.bancrofti     GCTGATCAGGACGATCTCGGGAAAGGAGTTGGTGATAAGAAGGACGAAAGTCTTAAAACC 420
O.volvulus      GCTGATCAGGATGATCTCGGAAAAGGAGTCGGCGCGAAAAAGGATGAAAGCCTGAAAACT 420
D.immitis       GCTGGTCAGGACGATCTAGGTGATGGTGTTGGCGATATAAAGGATGAAAGCCTGAAAACC 420
                **** *** ** ***** **  * ** ** ** *  *  ***** ***** ** *****

B.phangi        GGTAATGCTGGTGCCCGTGTTGCATGTGGTATTGTTGCCGTTAGTGCTGCTTCTTGA 477
B.malayi        GGTAATGCTGGTGCCCGTGTTGCATGTGGTATTGTTGCCATTGGTGCTGCTTCTTGA 477
W.bancrofti     GGTAATGCTGGTGCTCGTGTTGCATGTGGTATTGTTGCCGTTAGTGCTGCTTCTTGA 477
O.volvulus      GGTAATGCTGGTGCTCGTGTTGCATGCGGTATTGTCGCTATTGGTGCTGCTTCCTAA 477
D.immitis       GTTAATGCTGGTCCTCGTGTTGCGTGTGGTATCATTGTATTAGTTCTTATATCATAA 477
                * ********** * ******** ** *****  * *   *   *  *   ** * *
```

Figure 21 Nucleotide sequence comparison of *Cu/Zn WbSOD* gene to intracellular Cu/Zn SOD of *B. pahangi, B. malayi, O. volvulus* and *D. immitis.*

Table 4 Comparison of cDNA sizes and homology of nucleotide and deduced amino acid sequences of *Cu/Zn WbSOD* gene against those from other organisms.

Organisms	Accession Number	Size of cDNA (bp)	% Homology	
			nucleotide	amino acid
C. elegans				
extracellular	AB003924	531	43	39
intracellular	L20135	477	63	66
O. volvulus				
extracellular	L13778	605	72	61
intracellular	X57105	477	86	87
D. immitis				
intracellular	AF004949	477	83	77
B. pahangi				
extracellular	X76283	600	89	81
intracellular	X76284	477	95	96
B. malayi				
intracellular	AY428604	477	96	95
Human				
extracellular	J02947	723	7	30
intracellular	AY049787	465	48	56
W. bancrofti (this study)	DQ377960	477	100	100

The identification of restriction enzyme sites within of complete *Cu/Zn WbSOD* gene was shown in Figure 22.



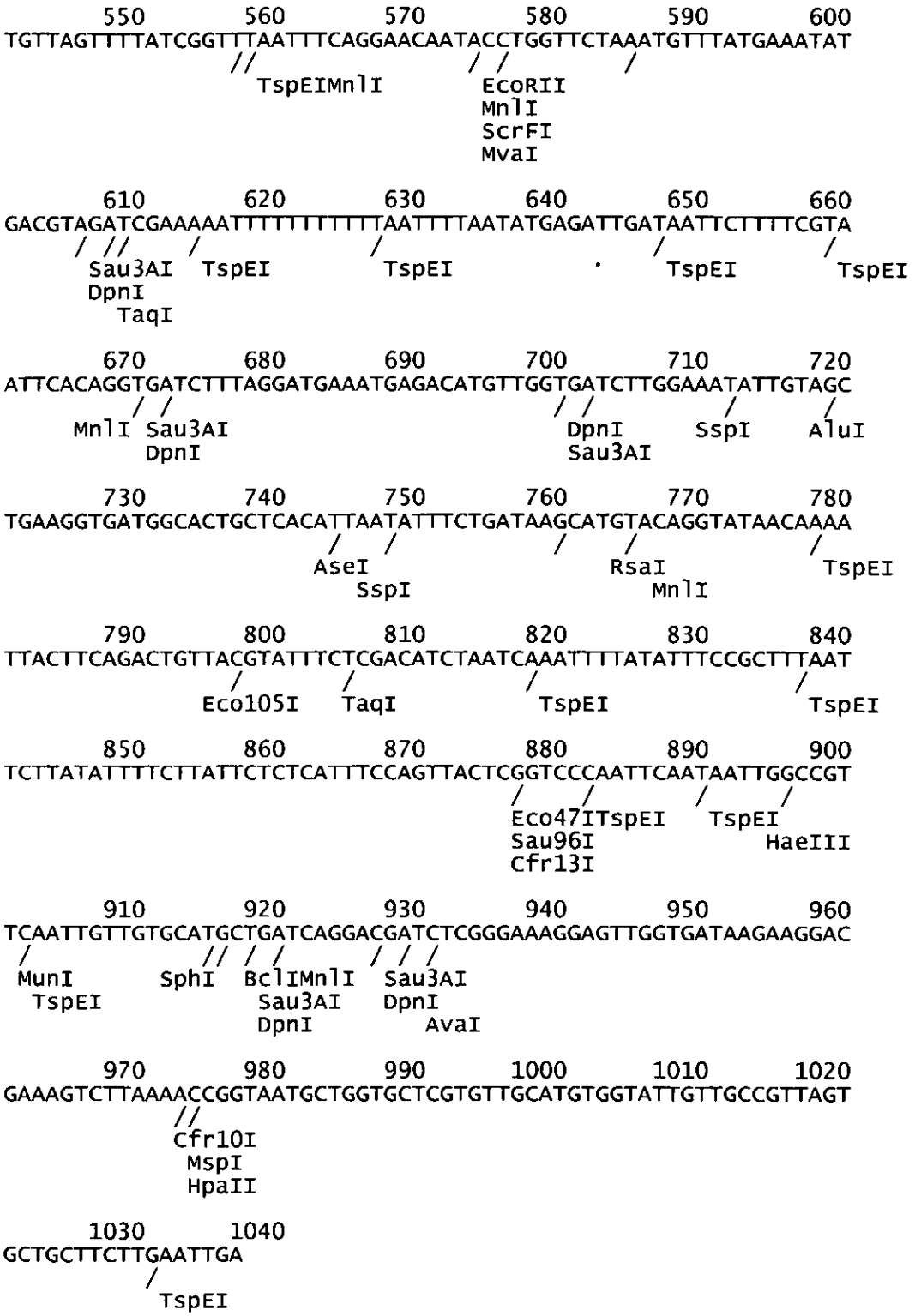


Figure 22 Restriction enzyme sites of complete *Cu/Zn WbSOD* gene.

[GENETYX-WIN: Search Restriction Site]
Date : 2005.07.18
Filename : comple wbsod.txt
Sequence Size : 1037
Sequence Position: 1 - 1037

[Linear]

Res. Ezm.	Recog. Seq.	Count:	Sequence Pos.					
AluI	AGCT	(2):	18	718				
AseI	ATTAAT	(1):	743					
AvaI	CYCGRG	(1):	931					
BclI	TGATCA	(1):	918					
Cfr10I	RCCGGY	(1):	973					
Cfr13I	GGNCC	(3):	448	484	877			
DpnI	GATC	(5):	607	671	701	919	928	
DraI	TTTAAA	(2):	58	327				
Eco105I	TACGTA	(1):	795					
Eco47I	GGWCC	(3):	448	484	877			
EcoRII	CCWGG	(1):	575					
EcoT22I	ATGCAT	(1):	435					
HaeIII	GGCC	(1):	895					
HpaII	CCGG	(2):	386	974				
MnlI	CCTG/CAGG	(6):	575	64	564	666	767	922
MspI	CCGG	(2):	386	974				
MunI	CAATTG	(1):	902					
MvaI	CCWGG	(1):	575					
NciI	CCSGG	(1):	385					
RsaI	GTAC	(2):	236	764				
Sau3AI	GATC	(5):	607	671	701	919	928	
Sau96I	GGNCC	(3):	448	484	877			
ScrFI	CCNGG	(2):	385	575				
SphI	GCATGC	(1):	912					
SspI	AATATT	(3):	180	710	746			
StyI	CCWWGG	(1):	511					
TaqI	TCGA	(3):	54	609	805			
TspEI	AATT	(18):	70	82	125	141	279	525
			559	615	628	648	660	779
			819	838	883	891	903	1032

Selected Enzymes With No Recognized Positions

AatI	AatII	AccI	AflII	Alw44I	ApaI	BamHI	BanI
BanII	BanIII	BbrPI	BfrI	BglI	BglII	Bpu1102I	Bsh1236I
BsiWI	BsmI	BssHII	BstEII	BstXI	Cfr9I	Csp45I	CspI
DdeI	DraIII	Eam1104I	Eco47III	Eco52I	Eco81I	Eco0109I	EcoRI
EcoRV	EheI	FspI	HaeII	HhaI	Hin1I	HincII	HindIII
HinfI	HpaI	KpnI	MboII	MluI	MroI	MscI	NaeI
NarI	NcoI	NdeI	NheI	NotI	NruI	NspV	PacI
PpuMI	PstI	PvuI	PvuII	SacI	SacII	SalI	SanDI
ScaI	SfiI	SmaI	SpeI	SrfI	XbaI	XhoI	XhoII
XmnI							

W=A/T, R=A/G, M=A/C, K=T/G, Y=T/C/U, S=G/C, N=A/T/G/C,
H=not G, B=not A, V=not U/T, D=not C

5.2 Codon usage analysis

The codon usage from Cu/Zn WbSOD sequence was shown in Table 5 and the 159 codons (including the stop codon) were listed. Purines (A and G) were preferred for the first position of the codon (71.7% A+G *cf* 28.3% C+T), the second position was shared between purines and pyrimidine (C and T). In the third position of codons, purine and pyrimidine were found 32.7% A+G and 67.3% C+T, respectively. (Table 6)

Nucleotide level of AT base pairs were presented in gene sequences at higher frequencies than GC (59.5% A+T *cf* 40.5% G+C). (Table 6) Codon usage of the third position, A or T were used rather than codons ending in G or C. Example, amino acid Gly and Ala that have four-fold coding degeneracy XYg, a, t and c. The codon XYt was more frequently used.

Table 5 Codon usage of *Cu/Zn WbSOD* gene.

AmAcid	Codon	Number	AmAcid	Codon	Number	AmAcid	Codon	Number
Gly	GGG	2	Glu	GAG	0	Val	GTG	1
Gly	GGA	4	Glu	GAA	6	Val	GTA	3
Gly	GGT	15	Asp	GAT	9	Val	GTT	8
Gly	GGC	3	Asp	GAC	2	Val	GTC	0
Ala	GCG	0	Arg	AGG	0	Lys	AAG	3
Ala	GCA	3	Arg	AGA	1	Lys	AAA	6
Ala	GCT	9	Ser	AGT	5	Asn	AAT	8
Ala	GCC	1	Ser	AGC	0	Asn	AAC	1
Met	ATG	2	Thr	ACG	0	Trp	TGG	0
Ile	ATA	2	Thr	ACA	3	End	TGA	1
Ile	ATT	10	Thr	ACT	4	Cys	TGT	1
Ile	ATC	1	Thr	ACC	2	Cys	TGC	1

Table 5 (continue)

AmAcid	Codon	Number	AmAcid	Codon	Number	AmAcid	Codon	Number
End	TAG	0	Leu	TTG	1	Ser	TCG	0
End	TAA	0	Leu	TTA	4	Ser	TCA	2
Tyr	TAT	0	Phe	TTT	2	Ser	TCT	3
Tyr	TAC	2	Phe	TTC	1	Ser	TCC	0
Arg	CGG	0	Gln	CAG	3	Leu	CTG	0
Arg	CGA	1	Gln	CAA	1	Leu	CTA	0
Arg	CGT	3	His	CAT	8	Leu	CTT	2
Arg	CGC	0	His	CAC	1	Leu	CTC	2
Pro	CCG	0	Pro	CCT	1			
Pro	CCA	3	Pro	CCC	2			

Table 6 Analysis of the proportion in *Cu/Zn WbSOD* gene.

Cu/Zn WbSOD	Base proportion per gene							
	A	T	C	G	A+G	C+T	A+T	C+G
	0.289	0.305	0.164	0.242	0.531	0.469	0.595	0.405
position of the codon	Base proportion per position of the codon							
	A	T	C	G	A+G	C+T	A+T	C+G
First	0.302	0.113	0.170	0.415	0.717	0.283	0.415	0.585
Second	0.316	0.247	0.203	0.234	0.550	0.450	0.563	0.437
Thirth	0.252	0.553	0.120	0.075	0.327	0.673	0.805	0.195

6. Phylogenetic analysis of *Cu/Zn WbSOD* gene

The phylogenetic relationship of *Cu/Zn WbSOD* nucleotide sequences was achieved by PAUP program version 4.0. The nucleotide sequence alignment of Cu/Zn SOD sequences 7 taxa including *W. bancrofti*, *B. pahangi*, *B. malayi*, *D. immitis*, *O.volvulus*, *C. elegans* and human were performed using Clustal W. (Figure 23) The cladograms were created using maximum parsimony method. For maximum parsimony analysis, the heuristic search strategy was utilized to find the most parsimonious trees using the parameters as followed; the number of substitution types = 2 (HKY85 variant), transition/transversion ratio = estimate, distribution of rates at variable sites = gamma (discrete approximation) and shape parameter (alpha) = estimate. The bootstrap maximum parsimony was analyzed with 100 replicates, addition sequence = as-is and Branch-swapping algorithm = tree-bisection-reconnection (TBR), all minimal trees saved and zero branch lengths collapsed; all characters were assigned equal weight. The trees resulting from PAUP were illustrated in Figure 24. Phylogenetic analysis of *Cu/Zn WbSOD* gene against those from other organisms revealed that the gene was closely related to those of *B. malayi* and *B. pahangi*.

```
B.phangi        ATGAGTGCGAATCGAATAGCTGTGTTACGTGGCGATA---ATGTTAGTGGGATTATTCGA 57
B.malayi        ATGAGTGCGAATGCAATAGCTGTGTTACGTGGCGATA---ATGTTAATGGGATTATTCGA 57
W.bancrofti     ATGAGTGCAAATGCAATAGCTGTATTACGTGGTGATA---ATGTTAGTGGGATTATTCGA 57
D.immitis       ATGAGTGCAAGTGCGATAGCTGTATTACGTGGCGAGG---CTGTTAGTGGAATTATTCGA 57
O.volvulus      ATGAGTACAAACGCGATAGCAGTATTGCGTGGCGATA---CTGTTAGTGGAATTATTCGA 57
C.elegans       ATGTCGAACCGTGCTGTCGCTGTTCTTCGTGGAGAAA---CTGTTACCGGTACTATCTGG 57
Human           ATGGCGACGAAGGCCGTGTGCGTGCTGAAGGGCGACGGCCCAGTGCAGGGCATCATCAAT 60
                ***              *    **  *    ** **       **    ** *  **

B.phangi        TTTAAACAGGAGAAAGAAGGATCGCCAACAACTATTAGTGGTGAAATCAAAGGTTTAACT 117
B.malayi        TTTAAACAGGAGAAAGAAGGATCGCCAACAACTATTAGTGGTGAAATCAAAGGTTTAACT 117
W.bancrofti     TTTAAACAGGAAAAAGAAGGATTACCAACAACTATTAGTGGTGAAATCAAAGGTTTAACT 117
D.immitis       TTTAAACAGGACAAAGAAGGCTTTCCAACAACCGTTAATGGTGAAATCAAGGGTTTAACT 117
O.volvulus      TTTAAACAGGACAAGGAAGGCTTACCAACAACCGTTACTGGTGAAGTCAAAGGTTTGACT 117
C.elegans       ATCACACAGAAGTCCGAAAATGACCAGGCAGTTATTGAAGGAGAAATCAAGGGACTTACT 117
Human           TTCGAGCAGAAGGAAAGTAATGGACCAGTGAAGGTGTGGGGAAGCATTAAAGGACTGACT 120
                 *    *** *             *          *    **     * ** **  * ***

B.phangi        CCCGGTTTGCATGGTTTTCATGTACATCAATACGGTGATACTACAAATGGATGCATTTCT 177
B.malayi        CCCGGTTTGCATGGTTTTCATGTACATCAATACGGTGATACTACAAACGGATGTATTTCT 177
W.bancrofti     CCCGGTTTGCATGGTTTTCATGTTCATCAATACGGTGATACTACAAACGGATGCATTTCT 177
D.immitis       CCTGGTTTGCATGGTTNTCATATTCATCAATTTGGTGATACTACAAATGGTTGCGTTTCT 177
O.volvulus      CCTGGTTTACATGGTTTTCATATTCATCAGTATGGTGACACGACAAATGGGTGCATTTCT 177
C.elegans       CCCGGTCTTCATGGATTCCACGTTCACCAATATGGTGATTCCACCAACGGATGCATTTCT 177
Human           GAAGGCCTGCATGGATTCCATGTTCATGAGTTTGGAGATAATACAGCAGGCTGTACCAGT 180
                   **  * *****  *  **  * **  * *  ** **    **    ** **      *

B.phangi        GCTGGTCCACATTTCAATCCTTACAATAAAACACATGGCGGTCCAACCGATGAAATGAGA 237
B.malayi        GCTGGTCCACATTTCAATCCTTACAATAAAACACATGGCGGTCCAACCGATGAAATGAGA 237
W.bancrofti     GCTGGTCCACATTTCAATCCTTACAATAAAACACATGGCGGTCCAACCGATGAAATGAGA 237
D.immitis       GCTGGTCCGCATTTTAATCCTCACAATAAGAATCATGGCGGTCCAACTGATGAAATAAGA 237
O.volvulus      GCGGGTCCGCATTTCAATCCTTACAATAAAACCCATGGCGATCGAACTGATGAAATAAGA 237
C.elegans       GCCGGTCCACACTTCAATCCATTTGGAAAGACTCATGGTGGACCAAAATCCGAGATCCGT 237
Human           GCAGGTCCTCACTTTAATCCTCTATCCAGAAAACACGGTGGGCCAAAGGATGAAGAGAGG 240
                ** ***** ** ** *****       *  *  ** ** *  * **     **     *

B.phangi        CATGTTGGTGATCTTGGAAATATTGTGGCTGGAGCTGATGGCACTGCTCACATTGATATT 297
B.malayi        CATGTTGGTGATCTTGGAAATATTGTGGCTGGAGCTGATGGCACTGCTCACATTGATATT 297
W.bancrofti     CATGTTGGTGATCTTGGAAATATTGTAGCTGAAGGTGATGGCACTGCTCACATTAATATT 297
D.immitis       CATGTTGGTGATCTTGGAAATATTGAAGCTGGGGCTGATGCTACAGCCCACATTGATATT 297
O.volvulus      CATGTTGGTGATCTTGGAAATATTGAAGCTGGAGCCGATGGTACTGCTCACATTAGCATT 297
C.elegans       CACGTAGGCGATCTAGGAAATGTGGAAGCTGGAGCCGATGGAGTGGCAAAAATCAAGCTC 297
Human           CATGTTGGAGACTTGGGCAATGTGACTGCTGACAAAGATGGTGTGGCCGATGTGTCTATT 300
                ** ** ** **  * ** *** *   ****     ****     **  *  *     *

B.phangi        TCTGATAAACATGTACAGTTACTCGGTCCCAATTCAATAATTGGTCGTTCACTTGTTGTG 357
B.malayi        TCTGATAAGCATGTACAGTTACTCGGTCCCAATTCAATAATTGGCCGTTCAATTGTTGTG 357
W.bancrofti     TCTGATAAGCATGTACAGTTACTCGGTCCCAATTCAATAATTGGCCGTTCAATTGTTGTG 357
D.immitis       TCTGATCAGAATATACAGTTGCTTGGTCCAAATTTGCTAATTGGGCGTTCAATTGTTGTT 357
O.volvulus      TCCGATCAACATATACAGTTGCTGGGTCCGAATTCGATAATTGGCCGTTCAATTGTTGTA 357
C.elegans       ACCGACACGCTCGTCACGCTTTACGGTCCAAACACTGTCGTTGGCCGATCTATGGTTGTT 357
Human           GAAGATTCTGTGATCTCACTCTCAGGAGACCATTGCATCATTGGCCGCACACTGGTGGTC 360
                   **        *     *    **      *      *  **** **  *  * ** **

B.phangi        CATGCTGATCAAGACGATCTCGGGAAAGGAGTTGGTGACAAGAAGGACGAAAGTCTTAAA 417
B.malayi        CATGCTGATCAAGACGATCTCGGGAAAGGAGTTGGTGACAAGAAGGACGAAAGTCTTAAA 417
W.bancrofti     CATGCTGATCAGGACGATCTCGGGAAAGGAGTTGGTGATAAGAAGGACGAAAGTCTTAAA 417
D.immitis       CACGCTGGTCAGGACGATCTAGGTGATGGTGTTGGCGATATAAAGGATGAAAGCCTGAAA 417
O.volvulus      CATGCTGATCAGGATGATCTCGGAAAAGGAGTCGGCGCGAAAAAGGATGAAAGCCTGAAA 417
C.elegans       CATGCCGGACAAGACGACCTCGGCGAGGGAGTCGGAGACAAGGCAGAAGAGTCCAAGAAG 417
Human           CATGAAAAAGCAGATGACTTGGGCAAAGGTG---------GAAATGAAGAAAGTACAAAG 411
                ** *         ** **  * **  * ** *              ** **       **

B.phangi        ACCGGTAATGCTGGTGCCCGTGTTGCATGTGGTATTGTTGCCGTTAGTGCTGCTTCTTGA 477
B.malayi        ACCGGTAATGCTGGTGCCCGTGTTGCATGTGGTATTGTTGCCATTGGTGCTGCTTCTTGA 477
W.bancrofti     ACCGGTAATGCTGGTGCTCGTGTTGCATGTGGTATTGTTGCCGTTAGTGCTGCTTCTTGA 477
D.immitis       ACCGTTAATGCTGGTCCTCGTGTTGCGTGTGGTATCATTGTATTAGTTCTTATATCATAA 477
O.volvulus      ACTGGTAATGCTGGTGCTCGTGTTGCATGCGGTATTGTCGCTATTGGTGCTGCTTCCTAA 477
C.elegans       ACTGGAAACGCCGGAGCTCGTGCTGCCTGCGGTGTCATTGCTCTCGCTGCTCCCCAGTGA 477
Human           ACAGGAAACGCTGGAAGTCGTTTGGCTTGTGGTGTAATTGGGATCG------CCCAATAA 465
                ** *  ** ** **    ***   ** ** *** *  * *   *             * *
```

Figure 23 Nucleotide sequence alignment of *Cu/Zn WbSOD* gene against *Cu/Zn SOD* from 6 taxa containing *B. pahangi*, *B. malayi*, *D. immitis*, *O.volvulus*, *C. elegans* and human. Using program Clustal W.

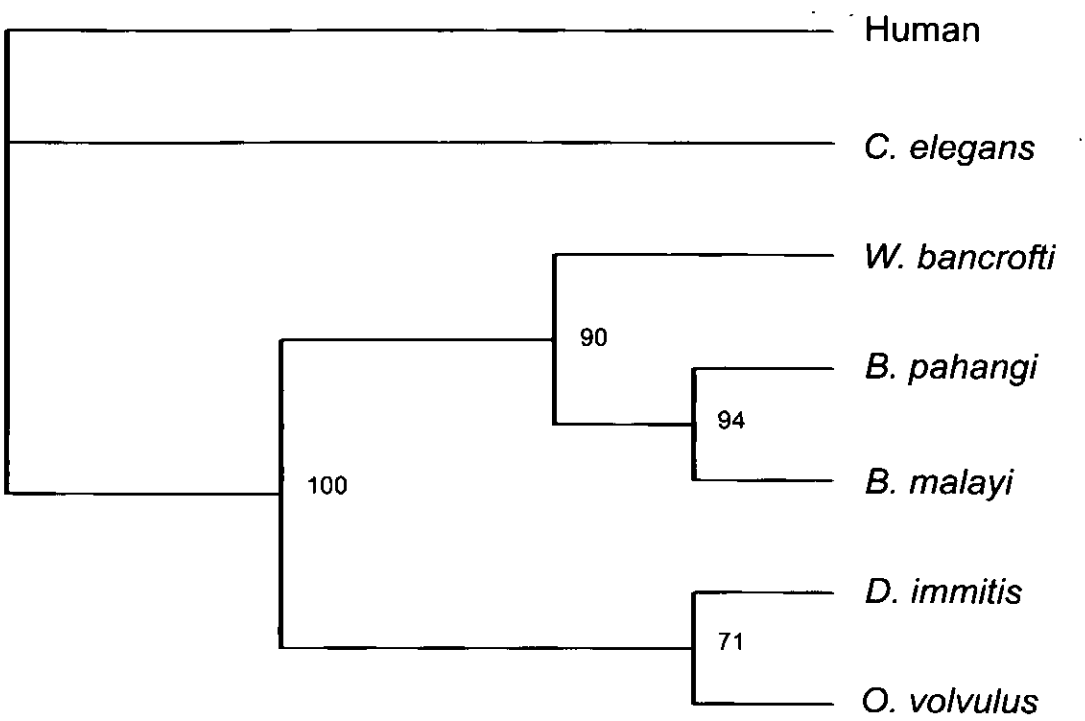


Figure 24 A representative parsimony cladogram of *Cu/Zn SOD* gene of *W. bancrofti*, *B. pahangi*, *B. malayi*, *D. immitis*, *O.volvulus*, *C. elegans* (outgroup) and human (outgroup) using the heuristic search of PAUP program version 4.0. Branches with more than 50% bootstrap support were shown. No significance is placed on the lengths of the branches connecting the species.

CHAPTER V
DISCUSSION

Superoxide dismutase have been presented in virtually all organisms including parasites, it is important scavenger of superoxide radicals which are released by immune effector cells as a host defense mechanism to kill invading parasites. Therefore, in these parasites which SOD is at the host-parasite interface, it is believed that the enzyme plays an important role in protecting parasites from the host response.[90-91]

In this study, the complete *Cu/Zn WbSOD* gene was cloned and analyzed of the nucleotide sequence data. The *Cu/Zn WbSOD* gene consisted of 1,037 nucleotides containing 4 exon and 3 intron regions. The predicted coding region (477 nucleotides) contains 158 amino acids. The calculated monomeric molecular weight of 16.34 kDa is similar to the values of 19 kDa, 18 kDa, 16 kDa and 19 kDa for the intracellular Cu/Zn SOD of *B. malayi*[72], *D. immitis*[75], *O. volvulus*[73] and *B. pahangi*[71], respectively.

The coding nucleotide sequences of the *Cu/Zn WbSOD* gene was 477 bp in length which corresponded to 158 amino acids. The data was similar to the partial *Cu/Zn WbSOD* gene that has been reported by Dabir and coworkers (2005).[92] Nucleotide level of *Cu/Zn WbSOD* gene, A and T were presented at higher frequencies than G and C. In addition, AT basepairs were more common at the third-codon position than GC base pairs. Therefore, this indicated that *Cu/Zn WbSOD* gene was bias for AT basepairs over GC nucleotides.

Based on the amino acid comparison and homology, it is likely that Cu/Zn WbSOD is the intracellular enzyme. However, the data presented in this study are not sufficient to conclude that the intracellular and extracellular Cu/Zn WbSOD are encoded from either the same or different gene(s). Nevertheless, upon comparison of nucleotide and amino acid contents between intracellular and extracellular SOD of *O. volvulus*, *B. pahangi*, *C. elegans* and human, the result revealed that the percent homology were (71:61), (89:83), (38:37) and (17:38), respectively. (Table 7) These implied that the intracellular and extracellular SOD may be expressed from the different genes or the enzymes are isozymes.

Table 7 The homology between extracellular and intracellular *SOD* genes and SOD enzymes from various organisms.

Organisms	Homology between extracellular and intracellular SOD genes (%)	Homology between extracellular and intracellular SOD enzymes (%)
O. volvulus	71	61
B. pahangi	89	83
C. elegans	38	37
human	17	38

According to the characteristics of Cu/Zn WbSOD amino acid compositions, the enzyme possessed three common conserved features of the SOD, the copper-zinc-binding site of six histidine and one aspartate residues (His-46, His-48, His-63, His-71, His-80, His-120, Asp-83),[80,88-89] the cysteine residues inferring the intrasubunit disulfide bridge (Cys-57, Cys-149), and the arginine residue which is necessary for guiding the superoxide to the active site (Arg-146).[80,88] These common conserved positions were also observed when the amino acid sequences of extracellular SOD were aligned. (Figure 25) These data confirmed that the *Cu/Zn WbSOD* gene encoded for the SOD enzyme. At present, there is no report of the drug resistance to SOD activity. Hence, these data will be beneficial for further study concerning mutagenesis of some important nucleotide residues that many affect the drug activity.

Phylogenetic trees of *Cu/Zn WbSOD* gene against other intracellular Cu/Zn SOD from various organisms revealed that the gene was closely related to those of *B. malayi* and *B. pahangi* and distantly related to those of human. The different between intracellular SOD of filaria and human lead to the possibility of using SOD as the potential drug target for anti-filarial drugs.

Figure 25 Amino acid sequence alignment of Cu/Zn Wb/SOD compared to extracellular Cu/Zn SOD of *B. pahangi, O. volvulus* and Human. Residues copper-zinc binding, disulfide bond and superoxide guiding-to-active-site were indicated in boxed.

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APPENDICES

APPENDICES

Reagent

1. Phosphate buffered saline (PBS buffer)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
H_2O (distilled water)	800 ml
Adjust pH 7.4 with HCl	
H_2O (distilled water) adjust volume to	1000 ml

2. LB broth

Bacto™ peptone	10 g
Yeast extract	5 g
NaCl	10 g
H_2O (distilled water) adjust volume to	1000 ml

3. LB-ampicillin agar plate

Bacto™ peptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
H_2O (distilled water)	1000 ml
Ampicillin 100 μg/ml	

4. IPTG 200 mg/ml

IPTG (Isopropyl-β-D-thiogalactopyranoside, dioxane free)	1 g
H_2O (distilled water) (sterile)	5 ml

Reagent

5. X-Gal 20 mg/ml

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	100 mg
N, N-dimethylformamide	5 ml

O. volvulus

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SeqA Name          Len(nt)  SeqB Name          Len(nt)  Score
=============================================================
1    Ov extra       606      2    O.vol cyto    477      71
=============================================================

Ov extra           ATGATTAATTCATTTATCGTCATCTTCTTATCATTCCTAATTTTCATCAATTATGCTAAT 60
O.vol cyto         ------------------------------------------------------------


Ov extra           TTAGTATGCGTCGAAGCTACACATGTGTACGGTAGAAGATCACATAGCAATGGAATGCAT 120
O.vol cyto         ------------------------------------------------------------


Ov extra           GGCAATGGAGCTAGAAGAGCAGTGGCAGTATTACGTGGTGATGCTGGTGTCAGTGGGATT 180
O.vol cyto         ----ATGAG--TACAAACGCGATAGCAGTATTGCGTGGCGATACTG---TTAGTGGAATT 51
                       ***    ** **  **  * ******** ***** *** ***   * ***** ***

Ov extra           ATTTATTTCCAACAGGGTAGTGGAGGTTCAATAACAACAATTTCTGGTTCAGTTAGTGGT 240
O.vol cyto         ATTCGATTTAAACAGGACAAGGAAGGCTTACCAACAACCGTTACTGGTGAAGTCAAAGGT 111
                   ***   **  ******  *  * *** * *  ******  ** *****  *** *  ***

Ov extra           TTAACACCTGGTTTGCATGGTTTTCATGTTCATCAGTATGGTGATCAAACAAACGGTTGT 300
O.vol cyto         TTGACTCCTGGTTTACATGGTTTTCATATTCATCAGTATGGTGACACGACAAATGGGTGC 171
                   ** ** ******** ************ ****************    ***** ** **

Ov extra           ACATCTGCCGGTGACCATTATAATCCTTTTGGTAAAACTCATGGTGGTCCAAATGACAGA 360
O.vol cyto         ATTTCTGCGGGTCCGCATTTCAATCCTTACAATAAAACCCATGGCGATCGAACTGATGAA 231
                   *  ***** ***   ****  *******    ****** ***** * ** ** ***   *

Ov extra           ATTAAACATATTGGTGATCTTGGAAATATTGTAGCTGGAGCTAATGGCGTTGCTGAAGTT 420
O.vol cyto         ATAAGACATGTTGGTGATCTTGGAAATATTGAAGCTGGAGCCGATGGTACTGCTCACATT 291
                   ** * **** ********************** *********  ****   **** *  **

Ov extra           TATATAAATAGTTATGACATAAAGTTACGGGGTCCACTTTCCGTAATTGGACATTCACTT 480
O.vol cyto         AGCATTTCCGATCAACATATACAGTTGCTGGGTCCGAATTCGATAATTGGCCGTTCAATT 351
                      **      * *  * *** **** * ******   ***  ******* * **** **

Ov extra           GTTGTACATGCAAATACGGACGATCTCGGACAAGGAACCGGCAATATGAGGGAAGAAAGT 540
O.vol cyto         GTTGTACATGCTGATCAGGATGATCTCGGAAAAGGAGTCGGCGCGAAAAAGGATGAAAGC 411
                   ***********  **  *** ********* *****  ****   *  * *** *****

Ov extra           TTGAAAACCGGTAATGCCGGCTCTCGTCTGGCATGTGGTGTTATTGGCATTGCTGCTGTA 600
O.vol cyto         CTGAAAACTGGTAATGCTGGTGCTCGTGTTGCATGCGGTATTGTCGCTATTGGTGCTGCT 471
                   ******* ******** **  ***** * ***** *** ** * *  **** *****

Ov extra           TCTTAA 606
O.vol cyto         TCCTAA 477
                   ** ***


SeqA Name          Len(aa)  SeqB Name          Len(aa)  Score
=============================================================
1    OVintra       158      2    OVextra       201      61

OVintra            ------------------------------------MSTN----AIAVLRGDT-VSGI 17
OVextra            MINSFIVIFLSFLIFINYANLVCVEATHVYGRRSHSNGMHGNGARRAVAVLRGDAGVSGI 60
                                                       *  *    *:******: ****

OVintra            IRFKQDKEGLPTTVTGEVKGLTPGLHGFHIHQYGDTTNGCISAGPHFNPYNKTHGDRTDE 77
OVextra            IYFQQGSGGSITTISGSVSGLTPGLHGFHVHQYGDQTNGCTSAGDHYNPFGKTHGGPNDR 120
                   * *:*.. *  **::*.*.**********:***** **** *** *:**:.****. .*.

OVintra            IRHVGDLGNIEAGADGTAHISISDQHIQLLGPNSIIGRSIVVHADQDDLGKGVGAKKDES 137
OVextra            IKHIGDLGNIVAGANGVAEVYINSYDIKLRGPLSVIGHSLVVHANTDDLGQGTGNMREES 180
                   *:*:****** ***:*.*.: *.. .*:* ** *:**:*:****: ****:*.*  .::**

OVintra            LKTGNAGARVACGIVAIGAAS 158
OVextra            LKTGNAGSRLACGVIGIAAVS 201
                   *******:*:***::.*.*.*
```

B. pahangi

```
SeqA Name       Len(nt)  SeqB Name       Len(nt)  Score
==========================================================
1    BpCyto     477      2    Bpextra    600      89

BpCyto          ------------------------------------------------------------
Bpextra         ATGATGATTGCATCATTTGCTATCTTCTTATCACATATCATTTTTATCACTTATGCAACA 60

BpCyto          ------------------------------------------------------------
Bpextra         TCAAATCAACGATATTTTAAACCAAATATGCATAATAATATGACAATAACAATAAGAAGA 120

BpCyto          ---------ATGAGTGCGAATCGAATAGCTGTGTTACGTGGCGATAATG------TTAGT 45
Bpextra         ACAATAACGAAAACAGCAACAGCAATAGCAGTATTACATAGTGATAATGGCAATATTAAT 180
                          *  *  ** *    ****** ** **** * * *******      *** *

BpCyto          GGGATTATTCGATTTAAACAGGAGAAAGAAGGATCGCCAACAACTATTAGTGGTGAAATC 105
Bpextra         GGAACTATTCATTTTCAACAGGACAAAAAT------TCGACAACTATTAGTGGTGAAATC 234
                ** * *****  *** ******* *** *        * **********************

BpCyto          AAAGGTTTAACTCCCGGTTTGCATGGTTTTCATGTACATCAATACGGTGATACTACAAAT 165
Bpextra         AAAGGTTTAACTCCCGGTTTGCATGGTTTTCATGTACATCAATACGGTGATACTACAAAC 294
                ************************************************************

BpCyto          GGATGCATTTCTGCTGGTCCACATTTCAATCCTTACAATAAAACACATGGCGGTCCAACC 225
Bpextra         GGATGCATTTCTGCTGGTCCACATTTCAATCCTTACAATAAAACACATGGCGATCCAACC 354
                ************************************************** *******

BpCyto          GATGAAATGAGACATGTTGGTGATCTTGGAAATATTGTGGCTGGAGCTGATGGCACTGCT 285
Bpextra         GATGAAATGAGACATGTTGGTGATCTTGGAAATATTGTGGCTGGAGCTGATGGCACTGCT 414
                ************************************************************

BpCyto          CACATTGATATTTCTGATAAACATGTACAGTTACTCGGTCCCAATTCAATAATTGGTCGT 345
Bpextra         CACATTGATATTTCTGATAAACATGTACAGTTACTCGGTCCCAATTCAATAATTGGTCGT 474
                ************************************************************

BpCyto          TCACTTGTTGTGCATGCTGATCAAGACGATCTCGGGAAAGGAGTTGGTGACAAGAAGGAC 405
Bpextra         TCACTTGTTGTGCATGCTGATCAAGACGATCTCGGGAAAGGAGTTGGTGACAAGAAGGAC 534
                ************************************************************

BpCyto          GAAAGTCTTAAAACCGGTAATGCTGGTGCCCGTGTTGCATGTGGTATTGTTGCCGTTAGT 465
Bpextra         GAAAGTCTTAAAACCGGTAATGCTGGTGGCCGTGTTGCATGTGGTATTGTTGCCATCAGT 594
                *************************** ************************ * ***

BpCyto          GCTGCTTCTTGA 477
Bpextra         GCTTGA------ 600
                ***
```

```
SeqA Name          Len(aa)  SeqB Name          Len(aa)  Score
=================================================================
1    B.phangi Cy   158      2    Bp extra      199      83

B.phangi Cy       ------------------------------------------MSANRIAVLRGDN--VS 15
Bp extra          MMIASFAIFLSHIIFITYATSNQRYFKPNMHNNMTITIRRTITKTATAIAVLHSDNGNIN 60
                                                             :*. ****:.**  :.

B.phangi Cy       GIIRFKQEKEGSPTTISGEIKGLTPGLHGFHVHQYGDTTNGCISAGPHFNPYNKTHGGPT 75
Bp extra          GTIHFQQDK--NSTTISGEIKGLTPGLHGFHVHQYGDTTNGCISAGPHFNPYNKTHGDPT 118
                  * *:*:*:*....*********************************************.**

B.phangi Cy       DEMRHVGDLGNIVAGADGTAHIDISDKHVQLLGPNSIIGRSLVVHADQDDLGKGVGDKKD 135
Bp extra          DEMRHVGDLGNIVAGADGTAHIDISDKHVQLLGPNSIIGRSLVVHADQDDLGKGVGDKKD 178
                  ************************************************************

B.phangi Cy       ESLKTGNAGARVACGIVAVSAAS 158
Bp extra          ESLKTGNAGGRVACGIVAISA-- 199
                  *********.********:**::
```

C. elegans

```
SeqA Name          Len(nt)  SeqB Name          Len(nt)  Score
=================================================================
1    C.eleg intra   477      2    C.eleg extra   531      38
```

```
C.eleg intra      ------------------------------------------------------ATG 3
C.eleg extra      ATGAAAACTCGTGTTGTTTTAATTCTGGCTCTCTCCGTTTGCATTGAAGCCGCTTCCGAA 60

C.eleg intra      TCGAACCGTGC---TGTCGCTGTTCTTCGTGGAGAAACTGTTACCGGTACTATCTGGATC 60
C.eleg extra      GTGATTCGTGCACGTGCCTACATTTTCAAAGCTGAAGCTGGAAA-AATTCCAACTGAATT 119
                    **  *****   ** *    ** *    *  *** ***  *    * * * *** **

C.eleg intra      ACACAGAAGT-CCGAAAATGACCA----GGCAGTTAT-TGAAG---GAGAAATCAAG--- 108
C.eleg extra      GATTGGAACTATTGATTTTGATCAATCTGGAAGTTTTCTGAAGCTTAACGGATCGGTTTC 179
                       *** *   **   *** **    ** **** * *****    *   ***

C.eleg intra      -GGACTTACTCCCGGTCTTCATGGATTCCACGTTCACCAATATGGTGATTCCACCAACGG 167
C.eleg extra      CGGATTAGCAGCTGGAAAACATGGATTCCATATTCATGAAAAAGGAGATACTGGAAATGG 239
                   *** *  *  * **    ***********  ****  ** * ** *** *    ** **

C.eleg intra      ATGCATTTCTGCCGGTCCACACTTCAATCCATTTGGAAAGACTCATGGTGGACCAAAATC 227
C.eleg extra      TTGTCTAAGTGCTGGTGGACATTATAATCCTCATAAGCTGAGCCATGGTGCACCAGATGA 299
                   **  *   *** *** *** *  *****   *     **  ******* **** *

C.eleg intra      CGAGATCCGTCACGTAGGCGATCTAGGAAATGTGGAAGCTGGAGCCGATGGAGTGGCAAA 287
C.eleg extra      CTCGAACAGACATATTGGAGATTTGGGAAATATTGAGTCACCGGCTTCCGGAGACACATT 359
                  *  ** * * **  * ** *** * ****** * **  *    **    ****   **

C.eleg intra      AATCAAGCTCACCGACACGCTCGTCACGCTTTACGGTCCAAACACTGTCGTTGGCCGATC 347
C.eleg extra      AATTTCAGTTTCCGACTCTCTTGCCTCATTAAGTGGACAATATTCAATCATTGGCCGAAG 419
                  ***     *  ***** * ** * * *  *    ** * * *  *  ** ********

C.eleg intra      TATGGTTGTTCATGCCGGACAAGACGACCTCGGCGAGGGAGTCGGAGACAAGGCAGAAGA 407
C.eleg extra      TGTGGTTATTCATGAAAAAACTGATGACTT------GGGACGCGG---TACTTCAGACCA 470
                  * ***** ******    *   ** *** *      ****  ***    *   ****  *

C.eleg intra      GTCCAAGAAGACTGGAAACGCCGGAGCTCGTGCTGCCTGCGGTGTCATTGCTCTCG-CTG 466
C.eleg extra      ATCAAAAACAACTGGAAATGCTGGAAGCCGACTTGCATGTGGAACTATCGGTACCGTCTG 530
                   ** ** *  ******** ** ***   **   *** ** **    ** * *  ** ***

C.elegintra       CTCCCCAGTGA 477
C.elegextra       A---------- 531
```

```
SeqA Name            Len(aa)  SeqB Name            Len(aa)  Score
=================================================================
1    C.elegan intra   161      2    C.eleg extra     176      37
```

```
C.elegan intra    MSNRAVAVLRG----ETVTGTIWITQ---KSEN-------------DQ----AVIEGEIK 36
C.eleg extra      MKTRVVLILALSVCIEAASEVIRARAYIFKAEAGKIPTELIGTIDFDQSGSFLKLNGSVS 60
                  *..*.* :*      *:.: .*       *:*              **      ::*.:.

C.elegan intra    GLTPGLHGFHVHQYGDSTNGCISAGPHFNPFGKTHGGPKSEIRHVGDLGNVEAGADGVAK 96
C.eleg extra      GLAAGKHGFHIHEKGDTGNGCLSAGGHYNPHKLSHGAPDDSNRHIGDLGNIESPASGDTL 120
                  **:.* ****:*: **: ***:*** *:**.  :**.*... **:*****:*: *.* :

C.elegan intra    IKLTDTLVTLYGPNTVVGRSMETVVHAGQDDLGEGVGDKAEESKKTGNAGARAACGVIAL 156
C.eleg extra      ISVSDSLASLSGQYSIIGRS--VVIHEKTDDLGRGTSD---QSKTTGNAGSRLACGTIGT 175
                  *.::*:*.:* *  :::*** ..*:*   ****.*..*.:.:**.*****:* ***.*.

C.elegan intra    AAPQS 161
C.eleg extra      V---- 176
                  .:..:
```

Human

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	Human,intra	465	2	human,extra	723	17

```
Human,intra     ------------------------------------------------------------
Human,extra     ATGCTGGCGCTACTGTGTTCCTGCCTGCTCCTGGCAGCCGGTGCCTCGGACGCCTGGACG 60

Human,intra     ------------------------------------------------------------
Human,extra     GGCGAGGACTCGGCGGAGCCCAACTCTGACTCGGCGGAGTGGATCCGAGACATGTACGCC 120

Human,intra     ------------------------------------------------------------
Human,extra     AAGGTCACGGAGATCTGGCAGGAGGTCATGCAGCGGCGGGACGACGACGGCACGCTCCAC 180

Human,intra     -----ATGGCGACGAAGGCCGTGTGC-GTGCTGAAGGGCG-ACGGCCCAGTGCAG--GGC 51
Human,extra     GCCGCCTGCCAGGTGCAGCCGTCGGCCACGCTGGACGCCGCGCAGCCCCGGGTGACCGGC 240
                      ** *       *****  **   **** * * **  * **** * *     ***

Human,intra     ATCATCAATTTCGAGCAGAAGGAAAGTAATG----GACCAGTGAAGGTGTGG----GGAA 103
Human,extra     GTCGTCCTCTTCCGGCAGCTTGCGCCCCGCGCCAAGCTCGACGCCTTCTTCGCCCTGGAG 300
                 ** **   ***  ****   *         *    * *   *      * *    ***

Human,intra     GCATTAAAGGACTGACTGAAGGCCTGCA----TGGATTCCATGTTCATGAGTTTGGAGAT 159
Human,extra     GGCTTCCCGACCGAGCCGAACAGCTCCAGCCGCGCCATCCACGTGCACCAGTTCGGGGAC 360
                *  **   *  *   * ***   ** **     *   **** ** **  **** ** **

Human,intra     AATACAGCAGGCTGTACCAGTGCAGGTCCTCACTTTAATCCTCTATCCAGAAAACACGGT 219
Human,extra     CTGAGCCAGGGCTGCGAGTCCACCGGGCCCCACTACAACCCGCTGGCCGTGCCGCAC--- 417
                   *     *****        * ** ** ****  ** ** **  **       ***

Human,intra     GGGCCAAAGGATGAAGAGAGGCATGTTGGAGACTTGGGCAATGTGACTGCTGACAAAGAT 279
Human,extra     ---CCGCAG------------CACCCGGGCGACTTCGGCAACTTCGCGGTCCGCGACGGC 462
                   **  **             **    ** ***** *****  *  * *    * * *

Human,intra     GGTGTGGCCGATGTGTCTATTGAAGATTCTGTGATCTCACTCTCAGGAGACCATTGCATC 339
Human,extra     AGCCT--CTGGAG-GTACCGCGCCGGCCTGGCCGCCTCGCTCGCGGGCCCGCACTCCATC 519
                 *  *  * *  * **     *  *     *    *** *** * **    ** * ****

Human,intra     ATTGGCCGCACACTGGTGGTCCATGAAAAAGCAGATGACTTGGGCAAAGGTGGAAATGAA 399
Human,extra     GTGGGCCGGGCCGTGGTCGTCCACGCTGGCGAGGACGACCTGGGCCGCGGCGGCAACCAG 579
                 * *****  *  **** ***** *     *  ** *** *****   ** ** **  *

Human,intra     GAAAGTACAAAGACAGGAAACGCTGGAAGTCGTTTGGCTTGTGGTGTAATTGGGATCGCC 459
Human,extra     GCCAGCGTGGAGAACGGGAACGCGGGCCGGCGGCTGGCCTGCTGCGTGGTGGGCGTGTGC 639
                *  **     ***  ** ***** **  * **  **** **  * **  * **  *   *

Human,intra     CAATAA------------------------------------------------------ 465
Human,extra     GGGCCCGGGCTCTGGGAGCGCCAGGCGCGGGAGCACTCAGAGCGCAAGAAGCGGCGGCGC 699

Human,intra     -------------------------
Human,extra     GAGAGCGAGTGCAAGGCCGCCTGA 72
```

Human

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SeqA Name          Len(aa)  SeqB Name          Len(aa)  Score
==========================================================
1    Human,intra   154      2    human,extra   240      38
==========================================================

Human,intra       -MATKAVCVLKGDGP---------------------------------------------- 14
Human,extra       MLALLCSCLLLAAGASDAWTGEDSAEPNSDSAEWIRDMYAKVTEIWQEVMQRRDDDGTLH 60
                   :*  . *:* . *.

Human,intra       -----------------VQGIINFEQKESNGPVKVWGSIKGLTEGLHG----FHVHEFGD 53
Human,extra       AACQVQPSATLDAAQPRVTGVVLFRQLAPRAKLDAFFALEGFPTEPNSSSRAIHVHQFGD 120
                                    * *:: *.*  ... :..: :::*:.   :.    :***:***

Human,intra       NTAGCTSAGPHFNPLSRKHGGPKDEERHVGDLGNVTADKDGVADVSIEDSVISLSGDHCI 113
Human,extra       LSQGCESTGPHYNPLAVPHP------QHPGDFGNFAVR-DGSLWRYRAGLAASLAGPHSI 173
                   : ** *:***:***:  * ......:* **:**.:. .**       . . **:* *.*

Human,intra       IGRTLVVHEKADDLGKGGNEESTKTGNAGSRLACGVIGIAQ------------------- 154
Human,extra       VGRAVVVHAGEDDLGRGGNQASVENGNAGRRLACCVVGVCGPGLWERQAREHSERKKRRR 233
                  :**::***   ****:***: *.:.**** **** *:*:.

Human,intra       -------
Human,extra       ESECKAA 240
```

CURRICULUM VITAE

CURRICULUM VITAE

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