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**MOLECULAR CHARACTERISATION AND
PHYLOGENETICS OF MALAYSIAN GREEN AROWANA
(*Scleropages formosus*) IN PENINSULAR MALAYSIA**

M. Rizman-Idid¹, S. Rahman¹, H. A. Aziz, S. Muniandy² & M. Zakaria-Ismail¹

¹Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur

²Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur



ASIAN AROWANA (*Scleropages formosus*)



- Also known as dragon fish or “kelisa” (Malay)
- Highly prized ornamental fish (China, Japan, South East Asia)
- Value as much as USD\$ 200 per fingerling and between USD\$ 1,000 and 2,000 for 30cm length (Pouyaud et al., 2003)
- In Malaysia, its popularity began as freshwater game fish (Stuart, 1953; Locke, 1956).
- Popular as aquarium fish in 1960s (Yue et al., 2004).



ASIAN AROWANA (*Scleropages formosus*)



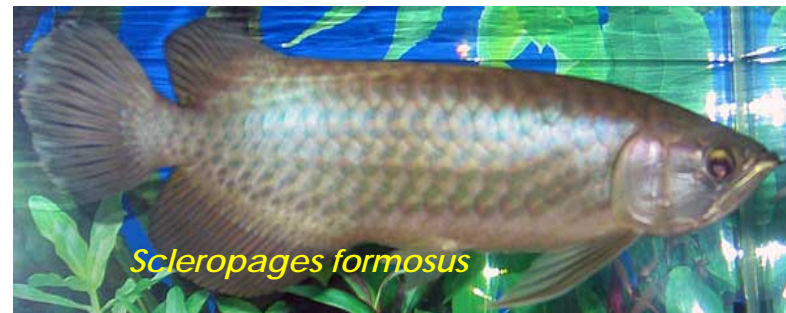
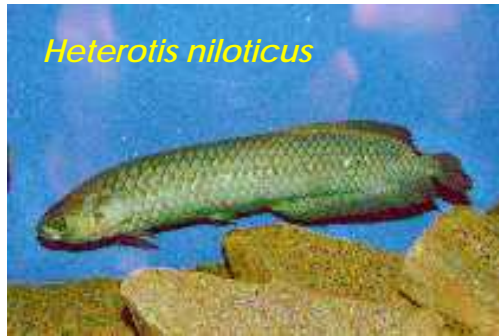
- The body of the Asian arowana is compressed, dorsal and anal fin are placed on the rear half of the body.
- They have large scales with varieties of colours and two barbels protruding from the lower jaw.



SYSTEMATICS OF ASIAN AROWANA



- Ancient “bony-tongue” family (Osteoglossidae)
- Extant representatives restricted to freshwater (Nelson, 1994).
- Order Osteoglossiformes
 - Family Osteoglossidae (4 genera)
 - *Osteoglossum* (South America)
 - *Arapaima* (South America)
 - *Heterotis* (Africa)
 - *Scleropages* (Southeast Asia , Australia)



GENUS *Scleropages*



- Genus *Scleropages* (3 species):
 - Northern barramundi (*Scleropages jardinii*)
 - Spotted barramundi (*Scleropages leichardti*)
 - Asian arowana (*Scleropages formosus*).
- Distribution pattern:
 - *Scleropages jardinii* (Northern Australia, Southern New Guinea)
 - *Scleropages leichardti* (Eastern Australia)
 - *Scleropages formosus* (Southeast Asia).

COLOUR VARIETIES OF *Scleropages formosus*



- Several types of *S. formosus* with different colour patterns inhabit separate regions of Southeast Asia
- Distributed into freshwater habitats during the Pleistocene glacial ages. (Goh & Chua, 1999)
- *S. formosus* have four natural colour varieties (Goh & Chua, 1999, Pouyaud et al., 2003):-
 - ❑ **Cross Back Golden** -Peninsular Malaysia
 - ❑ **Super Red** - West Kalimantan (Indonesia)
 - ❑ **Red Tail Golden** - Pekan Baru (Indonesia)
 - ❑ **Green** – Peninsular Malaysia, Indonesia, Myanmar, Thailand

DISTRIBUTION OF *S.formosus* IN PENINSULAR MALAYSIA



- Green and gold varieties only in the Peninsular Malaysia
- None has been reported from East Malaysia, Sabah and Sarawak (Suleiman, 2003).
- **Green variety** has been reported in some area in Peninsular Malaysia:-
 - Lake Tasek Bera (Pahang)
 - Endau River (Johor)
 - Terengganu drainage
- **Gold variety** is believed endemic to Bukit Merah Reservoir, in Perak, Malaysia. (Suleiman , 2003)
- Declining populations due to over-exploitations and destruction of their natural habitats.
- Placement of the species in the **Red List of IUCN** and **Appendix I in CITES**.

DISTRIBUTION OF *S.formosus* IN PENINSULAR MALAYSIA



Site: Bukit Merah Reservoir
Variety: Gold (extinct?)

Site: Trengganu
Variety: Green (extinct?)

Site: Lake Tasek Bera
Variety: Green (thriving)

Site: Endau River
Variety: Green (thriving)

MOLECULAR GENETIC STUDIES



- Molecular genetic techniques have been applied in captive breeding and conservation programs of this endangered species.
- Some of the techniques were based on RFLP, RAPD, AFLP, DNA fingerprinting, and microsatellite analysis.
- However, studies were more focused on captive stocks.
- Very few studies investigate the genetic variation of the natural and wild populations.

OBJECTIVES



- To describe the mitochondrial DNA sequence of natural population of green arowana from Lake Tasek Bera and Endau River (5'-end of partial *cyt-b* and complete *ND2* genes).
- To determine the accumulation of substitutions.
- To reconstruct the phylogenetic tree (*cyt-b* and *ND2* genes).
- To determine whether Tasek Bera population is different from Endau River population.



- Collection of samples (16 specimens) from:
 - ☐ Lake Tasek Bera (Pahang)
 - ☐ Endau river (Johor)
- Total of 23 sequences :
 - ☐ 15 green (10 Tasik Bera and 5 Endau Rompin)
 - ☐ 1 gold (Bukit Merah)
 - ☐ 2 red (Indonesian) by Kumazawa et al, (2004).
 - ☐ 5 outgroup sequences (*Arapaima gigas*, *Osteoglossum bicirrhosum*, *Osteoglossum ferreirai*, *Scleropages jardinii* and *Scleropages leichardti*).

LAKE TASEK BERA



- Peat swamp (Black water)
- Water is dark, tea coloured, and is black as seen by reflected light
- Acidic (pH4.3-6.45)
- Less water flow
- Vegetation :
 - *Pandanus heliococtus*
 - *Lepironia articulata*

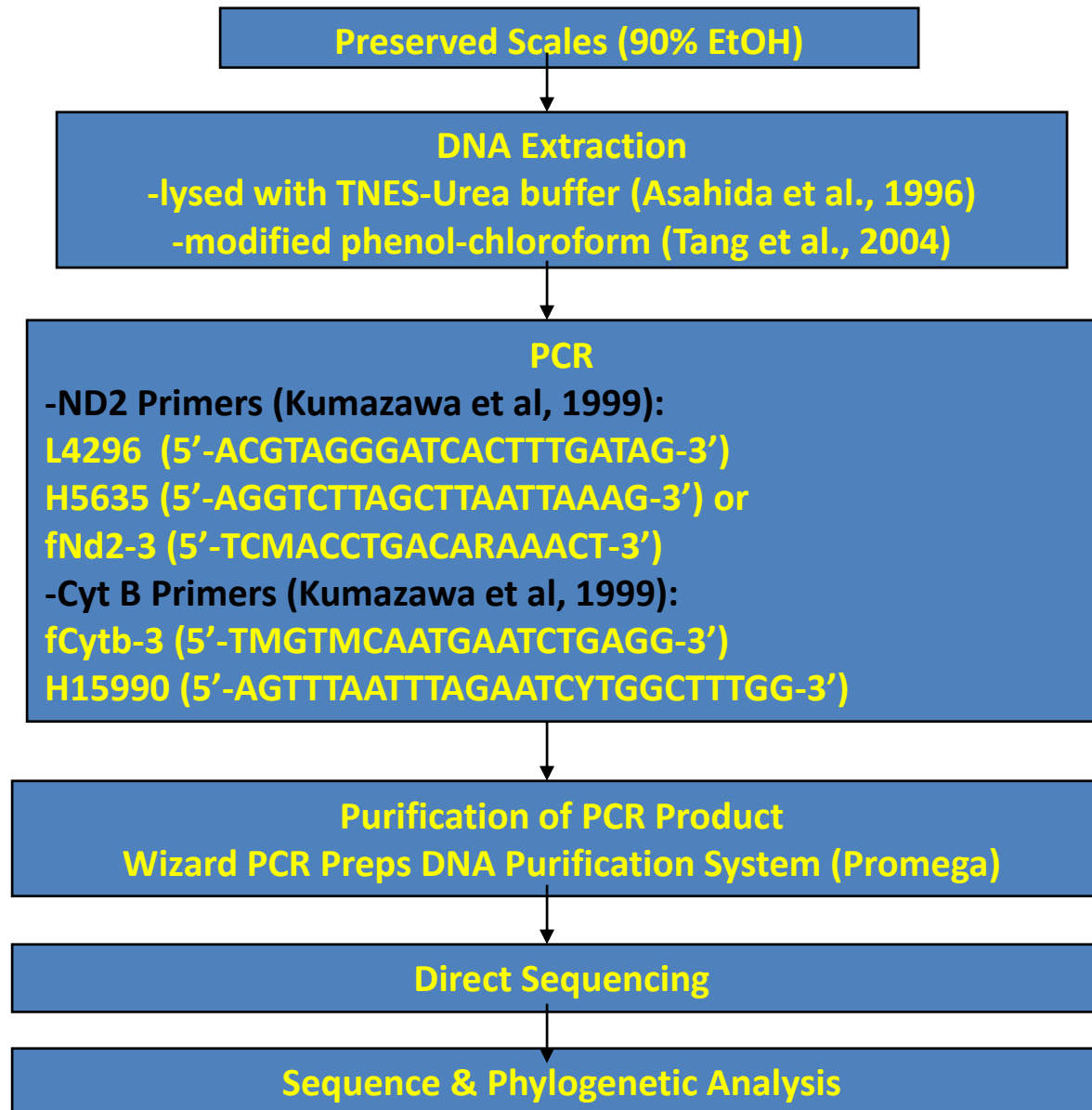
ENDAU RIVER



- Riverine system with pools
- Variable flow of water current
- Acidic (pH5.47-6.97)
- Typical rainforest vegetation



PCR AND SEQUENCING



MITOCHONDRIAL DNA SEQUENCES



Species name	Location (Species ID)	No of specimen	Accession No. (CytB)	Accession No. (ND2)
<i>S. formosus</i>	Endau Rompin	5	DQ864671-5	DQ864684-5 DQ147907-8 DQ147902
<i>S. formosus</i>	Bukit Merah	1	DQ864676	DQ147909
<i>S. formosus</i>	Tasik Bera	10	DQ864661-70	DQ864677-83 DQ864686 DQ147901 DQ147906
<i>S. formosus</i>	Indonesia	2	AB035234-5	AB035221
<i>S. jardinii</i>	Northern Australia	1	AB035236	AB035223
<i>S. leichardti</i>	Eastern Australia	1	AB035237	AB035224
<i>O. bicirrhosum</i>	South America	1	AB035238	AB035225
<i>O. ferreirai</i>	South America	1	AB035239	AB032526
<i>Arapaima gigas</i>	South America	1	AB035241	AB032528

BASE COMPOSITION



Base composition of cyt-b sequences

Species	T(U)	C	A	G
<i>S. formosus</i>	24.3*	35.8*	28.9*	11.0*
<i>S. jardinii</i>	26.5	34.9	25.7	12.9
<i>S. leichardti</i>	26.6	34.9	26.3	12.1
<i>O. bicirrhosum</i>	29.3	29.8	29.7	11.2
<i>O. ferreirai</i>	28.7	30.5	30.3	10.5
<i>A. gigas</i>	30.5	30.6	28.1	10.8

*average of 15 *S. formosus* (green arowana) sequences

BASE COMPOSITION



Base composition of ND2 sequences

Species	T(U)	C	A	G
<i>S. formosus</i>	20.9*	35.8*	31.7*	11.6*
<i>S. jardinii</i>	21.8	34.1	29.8	14.3
<i>S. leichardti</i>	21.8	34.6	32.5	11.1
<i>O. bicirrhosum</i>	23.2	32.2	34.5	10.1
<i>O. ferreirai</i>	23.3	32.0	34.8	9.9
<i>A. gigas</i>	27.2	29.9	32.2	9.7

*average of 15 *S. formosus* (green arowana) sequences

SEQUENCE VARIATION



cyt-b

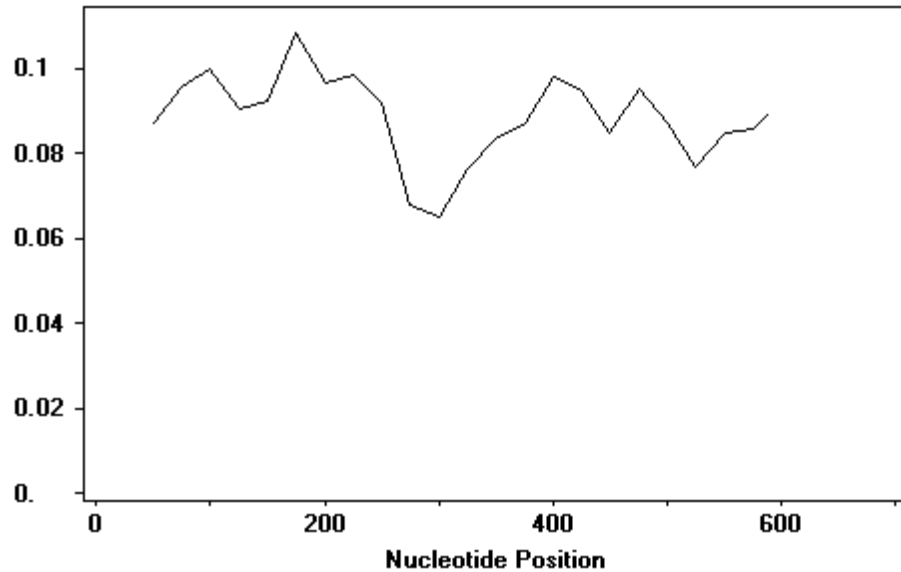
Type of site	Base frequency	(%)
Conserved	599	95.5
Variable	28	4.5
Singleton	13	2.1
Parsimony info	15	2.4

ND2

Type of site	Base frequency	(%)
Conserved	1012	97.2
Variable	29	2.8
Singleton	9	0.9
Parsimony info	20	1.9

Nucleotide frequency of variable sites of Cyt B (627bp) and ND2 (1041bp) from the alignment of 18 *S. formosus* sequences

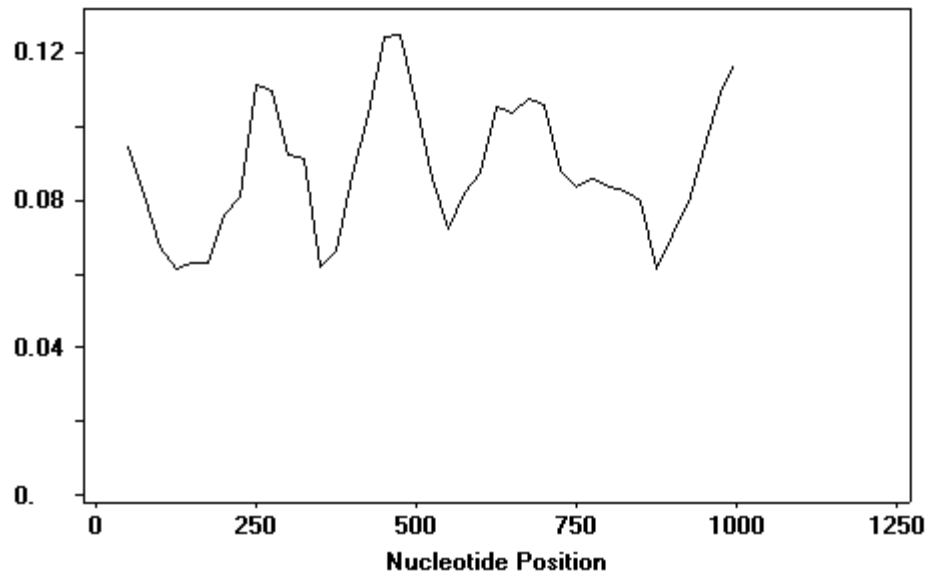
DISTRIBUTION OF SEQUENCE VARIATION



cyt b

Most variation found at position 50-250 and 400-600.

Average Pi nucleotide diversity = 0.088



ND2

Most variation found at position 80-90, 250-300, 470-510, 625-700 and 1000.

Average Pi nucleotide diversity = 0.09

AMINO ACID SEQUENCE VARIATION



Cyt-b=209 amino acids

Type of site	Amino acid frequency	(%)
Conserved	202	96.7
Variable	7	3.4
Singleton	6	2.9
Parsimony info	1	0.5

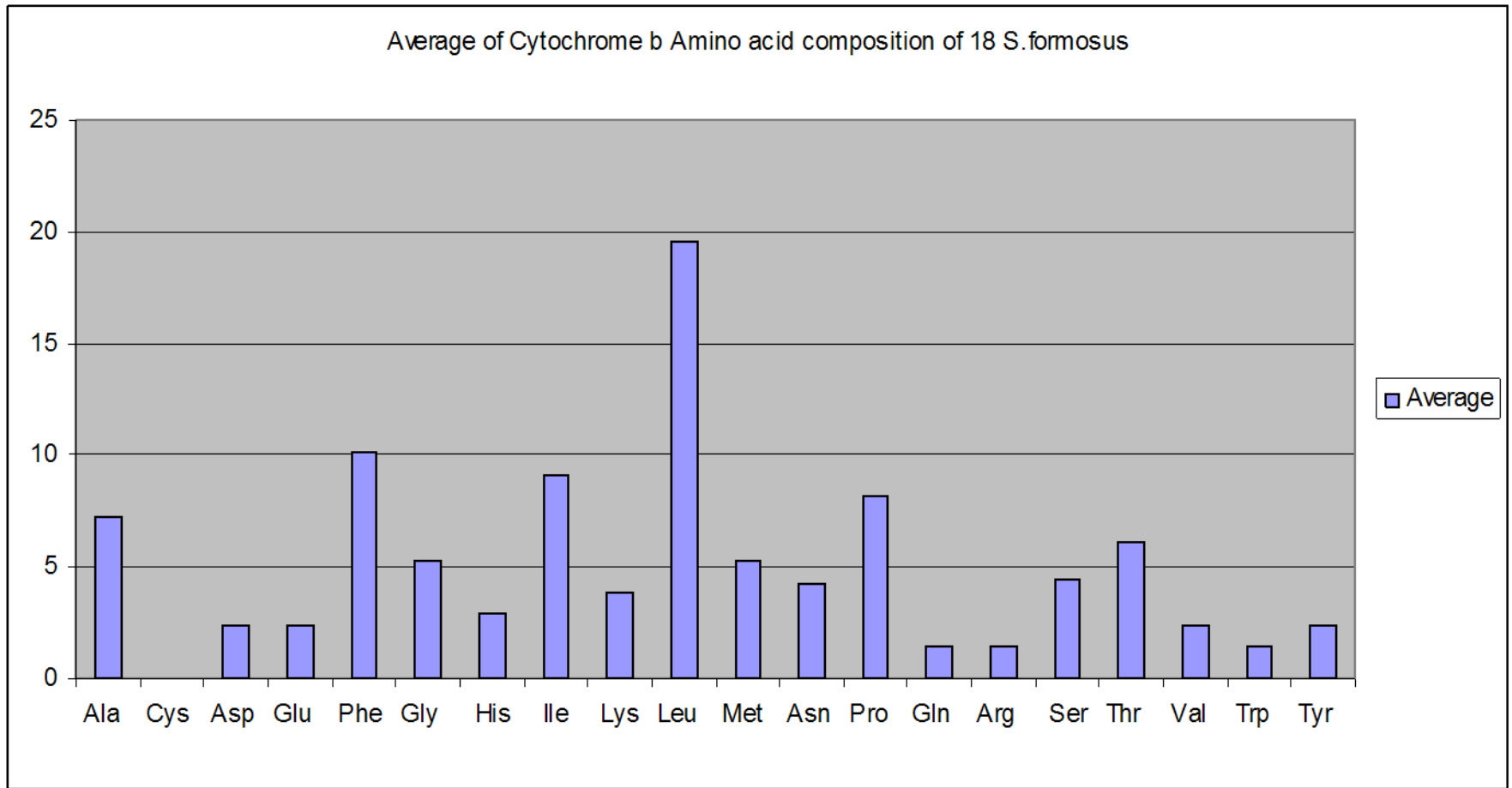
ND2 =347 amino acids

Type of site	Amino acid frequency	(%)
Conserved	339	97.7
Variable	8	2.3
Singleton	5	1.44
Parsimony info	3	0.9



- Amino acid composition of partial cyt-b sequences showed high percentage of leucine (19.56%) and phenylalanine (10.1%) residues.
- ND2 sequences also had high percentage of leucine (19.88%) and threonine (15.55%) residues.

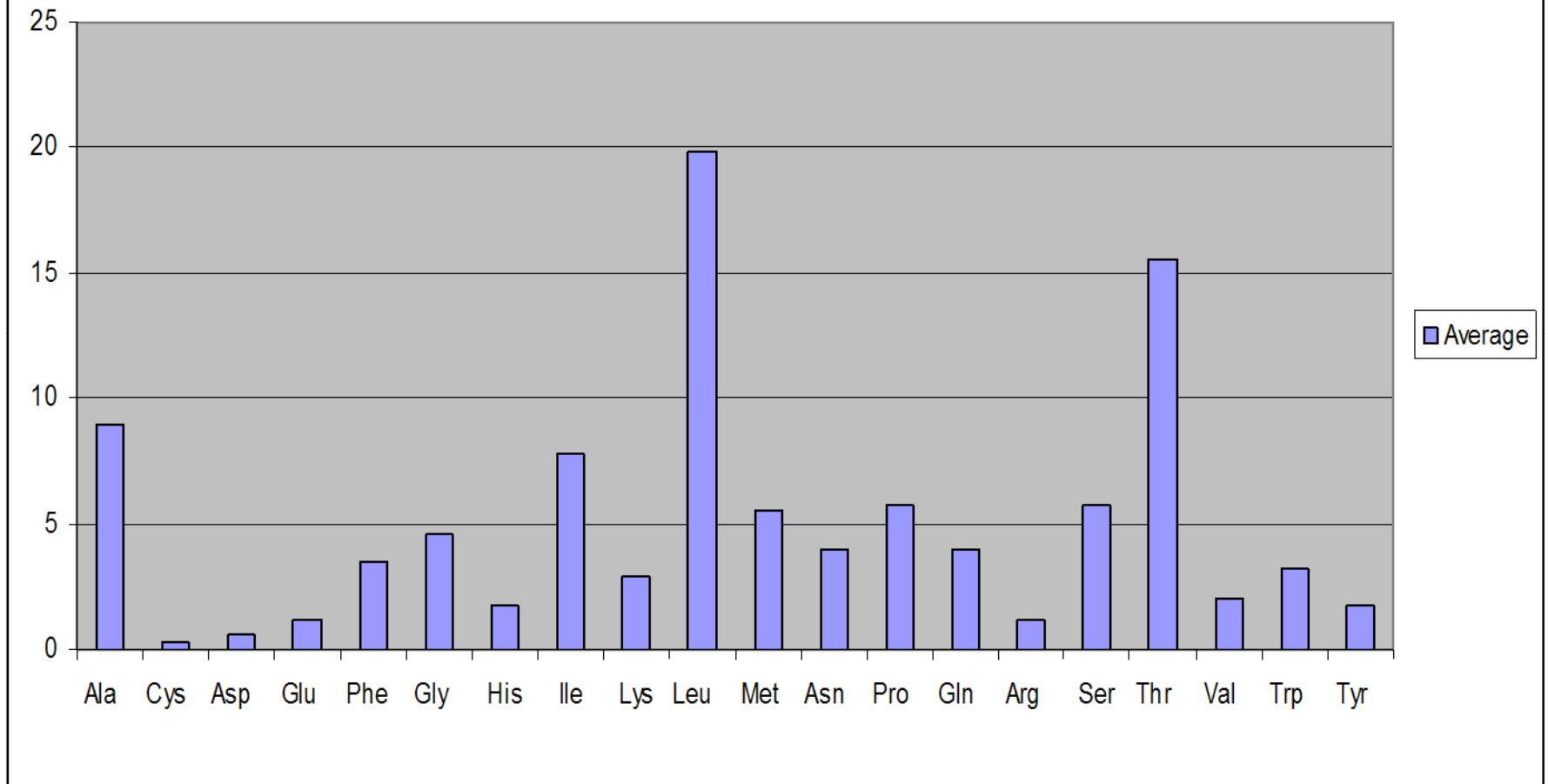
AMINO ACID COMPOSITION



AMINO ACID SEQUENCE VARIATION



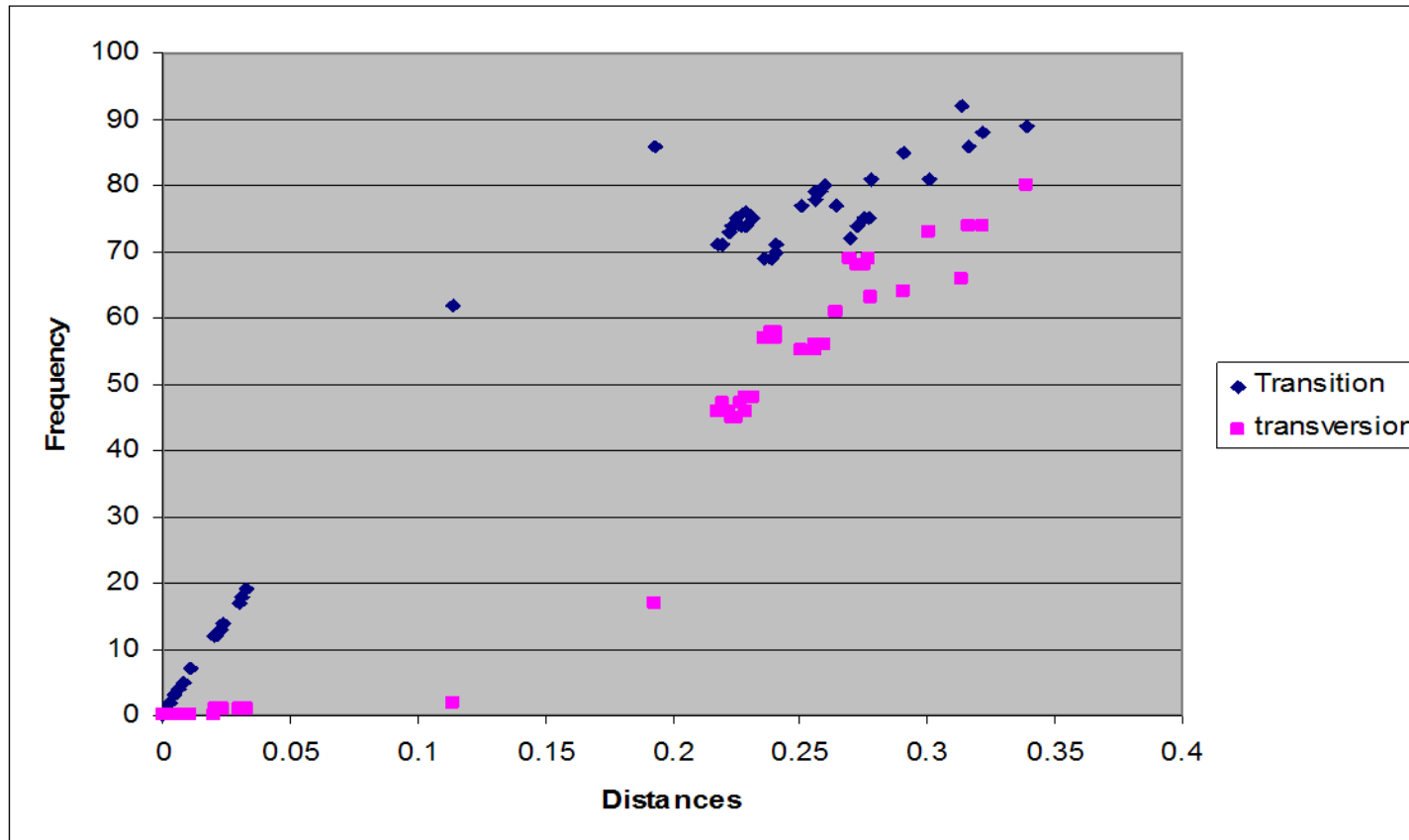
Average of ND2 Amino acid composition of 18 *S.formosus*



SUBSTITUTION PATTERN



- Transition and transversion rate against the genetic distance (Kimura-2-Parameter) in the cyt-b sequence of 18 *S. formosus* and 5 outgroup species.

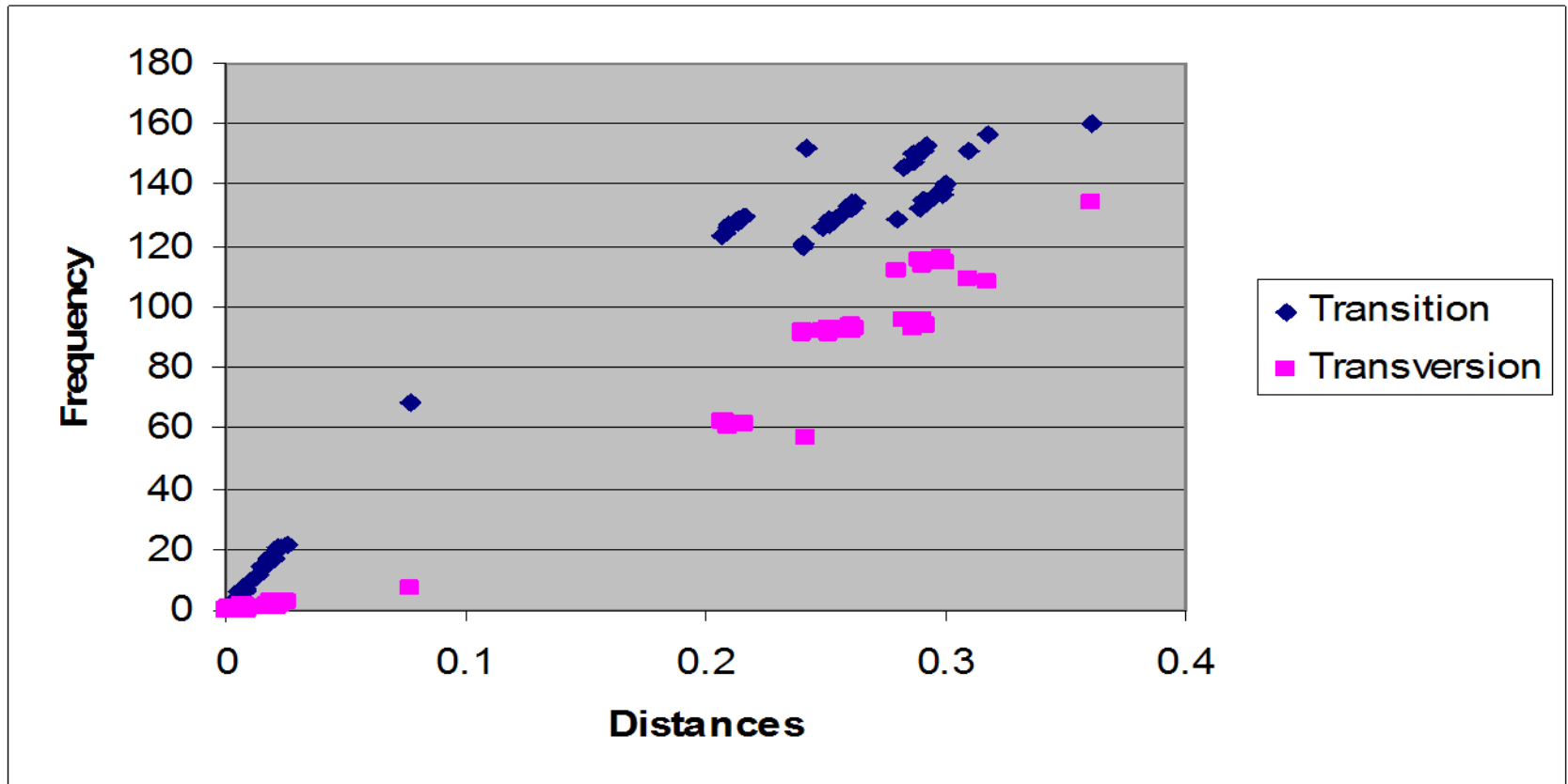


- Within *S. formosus*- more transitions than transversions
- Between genus/species- high level of transitions and transversions. Members of Osteoglossidae are highly divergent.

SUBSTITUTION PATTERN

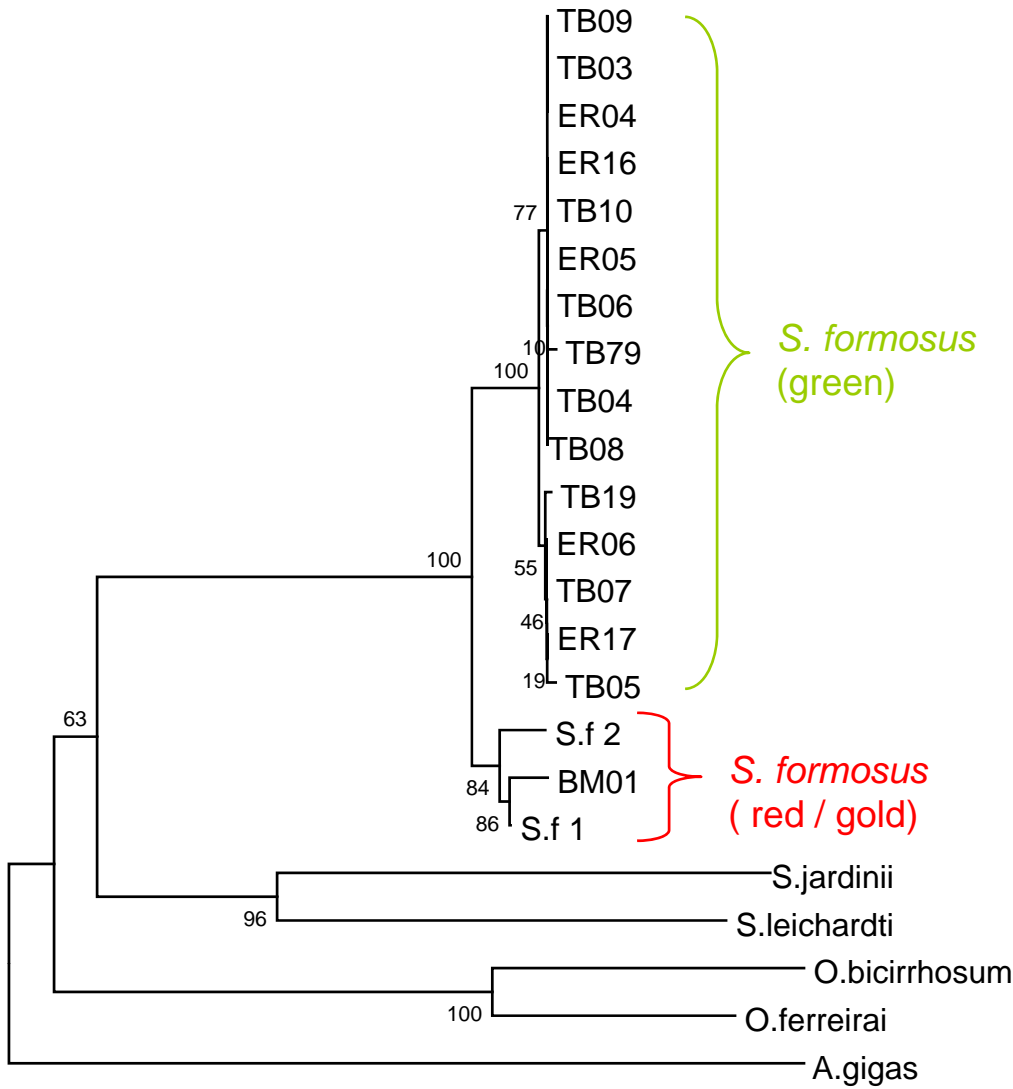


- Transition and transversion rate against the genetic distance (Kimura-2- parameter) in the ND2 sequence of 18 *S. formosus* and 5 outgroup species



- Similar substitution pattern as in cyt-b, but with higher substitution frequencies.
- For closely related species, the [transition/ transversion ratio](#) approximately 10:1. More divergent species have transitions/transversion ratio of 2:1.

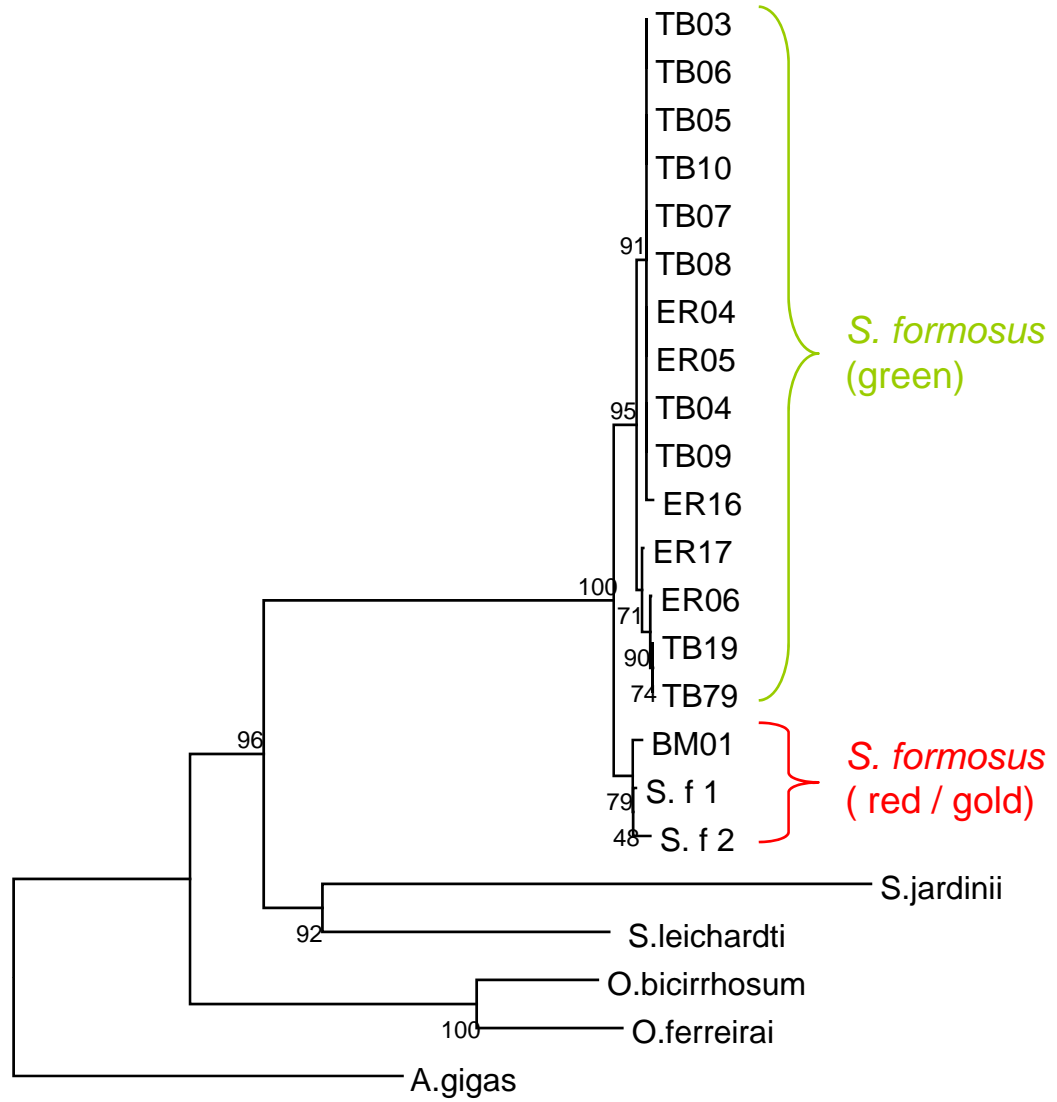
PHYLOGENETIC TREE



Neighbor Joining tree of 23 cytochrome b sequences (627 bp) using Kimura 2 parameter model (1000 bootstraps) and rooted with *A. gigas*

0.005

PHYLOGENETIC TREE



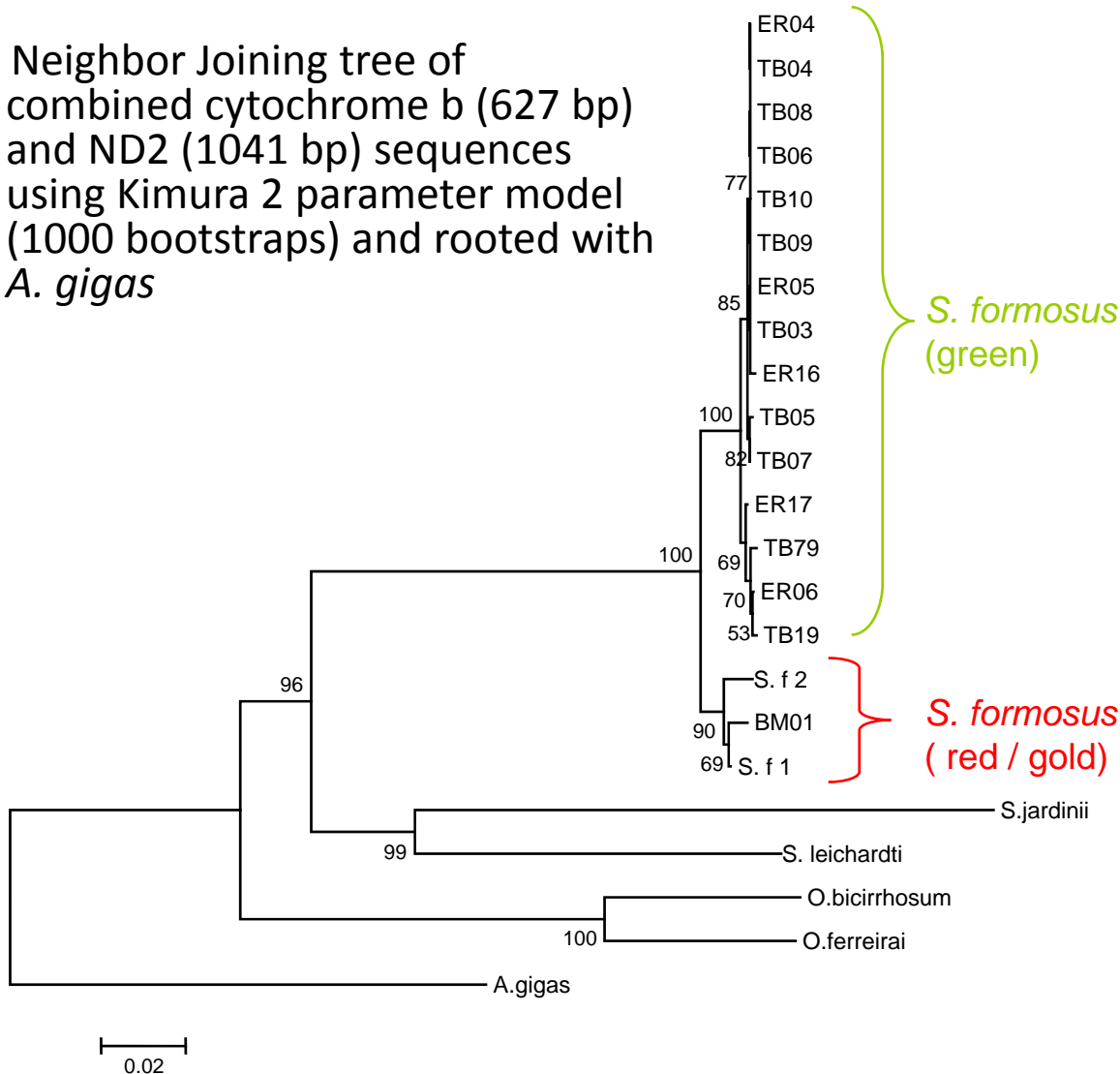
Neighbor Joining tree of 23 ND2 sequences (1041 bp) using Kimura 2 parameter model (1000 bootstraps) and rooted with *A. gigas*

H
0.005

PHYLOGENETIC TREE



Neighbor Joining tree of combined cytochrome b (627 bp) and ND2 (1041 bp) sequences using Kimura 2 parameter model (1000 bootstraps) and rooted with *A. gigas*



- Genus *Scleropages* is monophyletic
- *S. formosus* is monophyletic
- Green arowana is monophyletic
- No genetic differentiation between Tasek Bera and Endau Rompin populations
- Gold arowana more related to red arowana

DISCUSSION



- Both *cyt-b* and *ND2* genes were highly conserved with low sequence variation among *S. formosus*, especially within the green arowana population.
- Insufficient to distinguish the wild populations of green arowana between Tasek Bera and Endau Rompin.
- However, green arowana were genetically different from red/gold arowana.

GENETICALLY DIFFERENT COLOUR VARIETIES?



- Pouyaud et al., 2003 have suggested that colour varieties of *S. formosus* may represent different species.
- The species are
 - *Scleropages formosus* (Green varieties)
 - *Scleropages aureus* (Silver)
 - *Scleropages macrocephalus* (Red-tailed Golden)
 - *Scleropages legendrei* (Super Red)
- His analysis of partial cyt-b sequences (3' end) suggests a close relationship between the silver and the green varieties.
- Our study suggests that the Malaysian gold variety appears to be more related to the red varieties.

GENETICALLY DIFFERENT COLOUR VARIETIES?



- Strong evidence that the colour varieties may have diverged from at least 2 major lineages of *Scleropages* which are silver-green and red-gold.
- The further splitting of these 4 varieties into species as suggested by Pouyaud et al., 2003 may be premature as most of his measurements were made on cultivated specimens.
- More wild specimens are needed to resolve the splitting of the 4 colour varieties.

WILD POPULATIONS OF LAKE TASEK BERA AND ENDAU RIVER



- Genetic differentiation was not detected between the wild populations due to the slow mutation rate of the cyt-b and ND2 genes.
- Very few transversions and almost saturated transitions.
- Faster evolving genetic marker may potentially reveal genetic differentiation (eg- D-loop).
- Current study on morphometric and meristic of *S. formosus* revealed no significant difference between wild populations of Tasek Bera and Endau River.

CONCLUSION



- Variability for cyt-b and ND2 genes were low.
- Selective constraints maintained the function of the genes (selection for hydrophobic amino acid residues).
- Genus *Scleropages* is monophyletic.
- At least 2 major lineages of *Scleropages* were identified, the 1) silver-green and 2) red-gold lineages.
- Gold arowana from Bukit Merah Reservoir was more related to the red arowana from Indonesia than the Malaysian green arowana.
- Present study was unable to detect genetic differentiation between the wild populations of Lake Tasek Bera and Endau River.
- More representative samples are required for future studies.