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Functional analysis evolution history of gonadal soma-derived factor (*gsdf*) in black rockfish, *Sebastes schlegelii*

Abstract

As a teleost- and gonad-specific growth factor, *gsdf* has been indicated to play an important role in sex differentiation and determination. In this study, the complete open reading frame (ORF) of *gsdf* was isolated from black rockfish, *Sebastes schlegelii*. Bioinformatics analysis showed that there is a conserved transforming growth factor- β (TGF- β) domain located on the C-terminus of Gsdf. Multiple sequence alignments revealed that fish *gsdf* were highly conserved in TGF- β domain which suggested their functional conservatism. Synteny analysis provided evidence for the hypothesis that *gsdf* was originated from fish-specific genome duplication (FSGD). To further explore its function, the expression pattern was examined based on the RNA-seq data and the result showed that significantly sexually dimorphic expression existing between male and female individuals. These results suggested that *gsdf* might play an important role in maintenance of male characteristics in *Sebastes schlegelii*.

Keywords: *gsdf*, black rockfish, evolution history, gene expression

22 1. Introduction

23 Over the past decades, many studies have focused on sex
24 differentiation/determination systems in vertebrates. It has been revealed that *SRY/sry*,
25 the sex-linked testis-determining gene, triggers male differentiation in most mammals
26 [1, 2]. *Dmrt1* was found as an important sex-determination gene in several species
27 such as *Gallus gallus* and *Cynoglossus semilaevis* [3, 4]. In *Oryzias latipes*, the sex-
28 determination gene is *DMY* [5, 6]. Besides, the members of transforming growth
29 factor- β (TGF- β) family also contribute to sex-determination in fish [7-9]. For
30 example, *Amhy*, the Y-linked replication of *Amh*, function as the sex-determination
31 gene in *Odontesthes hatcheri* and *Oreochromis niloticus* which demonstrated the
32 significant role of *Amh/AmhR2* signaling pathway in sex determination in fish [8, 10].
33 The *gsdf* gene belongs to TGF- β family, which was found only in teleost fish [11-13].
34 In general, it is predominantly expressed in Sertoli cells and surrounding cells in
35 mature gonads, probably with some lineage-specific function [9, 11, 12]. Recent study
36 has confirmed that *gsdf* play an important role in the process of fish reproduction and
37 development. In *Oncorhynchus mykiss*, *gsdf* could enhance primordial germ cell and
38 spermatogonial proliferation [11]. Moreover, *gsdf* transcription is activated directly by
39 *dmy*, which established the autosomal *gsdf* as the first male sex initiator in *Oryzias*
40 *latipes* [14]. And in *Cynoglossus semilaevis*, the autosomal *gsdf* gene play a positive
41 role in germ differentiation and proliferation via influencing genes related to sex
42 differentiation [15].

43 Black rockfish (*Sebastes schlegelii*), an economic fish species, is cultured worldwide.
44 During the grow-out period, the growth rate of females is substantially faster than that
45 of males under the same culturing condition. Therefore, sex-controlled breeding is
46 very important and it is very meaningful to study mechanisms of gonadal sex
47 differentiation in this species. In the present study, a *gsdf* ortholog was isolated from
48 black rockfish and its distribution pattern in tissues was detected. Also, synteny
49 analysis was carried to discuss the origin of *gsdf*. Our results will facilitate to
50 understand the function of *gsdf* in black rockfish and help to the sex-controlled
51 breeding in the future.

52

53 **2. Methods**

54 *2.1 Sequence identification and bioinformatics analysis*

55 The cDNA sequence of *Ssgsdf* was obtained using local BLAST from transcriptome.
56 DNASTAR was used to analyze putative amino acid sequence, calculated molecular
57 weight, and theoretical isoelectric point. The signal peptide was analyzed by SignalP
58 v4.0 program. Protein domains were predicted using the Simple Modular Architecture
59 Research Tool [16, 17].

60 *2.2 Multiple sequence alignments and phylogenetic analysis*

61 All the other sequences were downloaded from NCBI websites (Table S1). Alignment
62 of putative amino acid sequences of black rockfish and other known vertebrates was
63 carried out by clustalX2 with the default parameters [18]. Phylogenetic tree was

64 constructed by neighbor-joining method and a bootstrap test with 1000 replicates
65 carried by MEGA 7.0 [19].

66 2.6 Synteny analysis

67 Synteny comparisons of the fragments harboring *gsdf* and flanking genes were
68 performed to test the genes' syntenic conservation. Flanking genes of *gsdf* used in the
69 synteny analysis were extracted from online genome databases. The genes were
70 mapped according to their relative locations in the chromosome for the synteny
71 analysis.

72 2.7 Expression analysis

73 To study the potential functions of *Ssgsdf*, the expression of *Ssgsdf* mRNA was
74 analyzed. The expression of *Ssgsdf* mRNA in different developmental stages and
75 different tissues were analyzed by TPM scores calculated from RNA-seq data.

76

77 3. Results

78 3.1 Sequence identification and analysis of *Ssgsdf*

79 The *gsdf* cDNA sequence of black rockfish was retrieved from the transcriptome
80 library. As shown in Fig. 1A, *gsdf* contains a 648 bp open reading frame (ORF)
81 encoding 216 amino acid residues. Black rockfish Gsdf (SsGsdf) has a calculated
82 molecular mass of 22.77 kDa and a theoretical pI of 5.06. An N-terminal signal
83 peptide formed by residues 1 to 19 was predicted by SignalP v4.0 program. Protein
84 domain prediction by SMART showed that the mature protein of *Ssgsdf* contains a

85 conserved TGF- β domain formed by residues 114-205 (Fig. 1B).

86 3.2 Multiple sequence alignments and phylogenetic analysis of *gsdf* orthologs

87 Multiple alignments of amino acid sequences of *Ssgsdf* and other teleost *gsdf* showed
88 that SsGsdf also presented seven conserved cysteine residues (Cys¹¹⁴, Cys¹¹⁵, Cys¹⁴³,
89 Cys¹⁴⁶, Cys¹⁷¹, Cys¹⁷², Cys²⁰⁴) that could be involved in the formation of the
90 characteristic cysteine knot motif, which is involved in intrachain disulfide bonds or
91 dimerization [20, 21]. The phylogenetic tree was constructed by was performed by
92 Bayesian method to show the relationship among different species. Two distinct
93 groups were separated in the phylogenetic tree. SsGsdf was clustered in the same
94 clade with teleost Gsdf. The clade formed by fish Gsdf was separated from the other
95 clade formed by other members of the TGF- β superfamily, which suggested that *gsdf*
96 was a unique member of this superfamily.

97 3.3 Synteny analysis of *gsdf* orthologs

98 As shown in Fig. 4, the *gsdf* genes and adjoining genes of several teleost species were
99 placed according to their relative locations on the scaffold or chromosome. All genes
100 near *gsdf* were highly conserved and shared the same direction in Tilapia, Amazon
101 molly, spotted gar and black rockfish. Comparison between three species revealed that
102 eight upstream genes (*gng10*, *btc*, *rxfp*, *slc45a2*, *amacr*, *sacrb2*, *nup54* and *ppef2*) and
103 eight downstream genes (*aff1*, *mrc1*, *klhl8*, *sdad1*, *ptpn13*, *mapk10*, *arhgap24* and
104 *esm1*) were conserved. In black rockfish, the downstream genes, *aff1* and *ptpn13*,
105 were replicated to produce two copies. Interestingly, only a few genes near *gsdf* gene

106 were found in cave fish, zebrafish and spotted gar. In these three species, the
107 transcriptional direction and the relative position relationship of some adjoining genes
108 were changed.

109 3.4 Tissue distribution pattern of *gsdf* mRNA

110 To analyze the presence of *gsdf* mRNA in black rockfish, we examined the expression
111 levels in ten different tissues of two-year post-hatching female and male individuals
112 by TPM scores. As shown in Fig. 5, large amounts of *gsdf* mRNA was observed in
113 adult testis, whereas the adult ovary expressed small amounts. Almost negligible
114 amplification for *gsdf* was detected in the non-gonadal tissues such as heart, liver,
115 spleen, kidney and gill.

116

117 4. Discussion

118 As a fish specific TGF- β family gene, *gsdf* is well known for its function in male sex
119 determination and differentiation [9, 12, 22-24]. In the present study, a *gsdf* ortholog
120 was isolated from *Sebastes schlegelii*, which encoded a 215-residue protein with a
121 single TGF- β domain. The deduced amino acid sequence indicated that it is relatively
122 well conserved in TGF- β domain. The cysteine residues of the TGF- β domain are
123 very conserved, indicating that Gsdf has similar functions in different species. As an
124 important signaling molecule, the actual form of Gsdf is a homo- or heterodimer of a
125 small carboxy-terminal. In TGF- β domain of SsGsdf, three disulfide bonds were
126 formed by Cys¹¹⁴-Cys¹⁷¹, Cys¹¹⁵-Cys¹⁷² and Cys¹⁴³-Cys²⁰⁴, and the intrachain disulfide

127 bond was formed by Cys¹⁴⁶ and a cysteine residue of another molecule [20]. Its N-
128 terminal signal peptide indicates that SsGsdF is secreted, suggesting that SsGsdF may
129 be secreted out of the cell and interact with the specific receptor molecules on the
130 surfaces of target cells.

131 During the long history of evolutionary process, many events occurred in fish genome,
132 such as whole-genome duplication (WGD), loss of chromosome fragments, gene
133 rearrangement and others [25, 26]. Previous studies revealed that *gsdf* is specific to
134 fish, which suggests that *gsdf* may originate from the fish-specific genome
135 duplication (FSGD) and be selectively dropped in the evolutionary process from fish
136 to tetrapods. Highly conserved genes around *gsdf* suggested that the functions of *gsdf*
137 and its adjoining genes tend to be constant. The different upstream and downstream
138 genes around *gsdf* between fishes suggested that gene rearrangement occurred in fish
139 genome. All these findings indicate the diversity of evolutionary events during the
140 evolutionary process of fish.

141 Up to date, the functions of *gsdf* were not elucidate clearly. Numerous studies in fish
142 revealed that *gsdf* only expressed in gonadal tissues, which suggested that its
143 functions may be restricted to the gonads. In this study, *SsgsdF* mRNA was only
144 expressed in the gonads, and the expression level in testis was significantly higher
145 than in the ovary. The expression pattern has obvious gender dimorphism. This result
146 was consistent with those in *Oncorhynchus mykiss*, *Oryzias latipes* and *Cynoglossus*
147 *semilaevis* [11, 12, 15]. The distinct gonad expression pattern in fish indicated that

148 *gsdf* may have a particular impact on gonad during the period of sex differentiation
149 and maintenance male characteristics.
150 In this study, *gsdf* was isolated and verified from *Sebastes schlegelii*. Bioinformatic
151 analysis revealed that *gsdf* was conserved in terms of potential domains and synteny
152 relationship. The expression pattern showed *Sebastes schlegelii gsdf* existed obvious
153 sexual dimorphism in adult. These results indicate that *Sebastes schlegelii gsdf* may
154 have essential functions in maintenance of male characteristics. Further studies are
155 necessary to illustrate the initiation mechanism of testis differentiation, which is
156 related to the regulatory mechanisms of *gsdf* expression.

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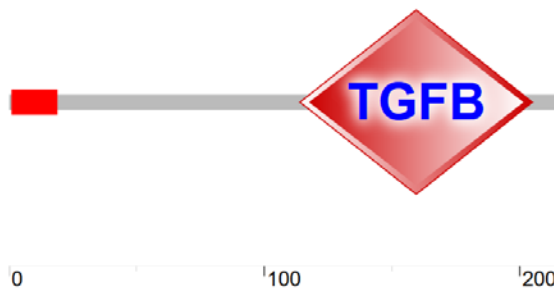
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235 **Figures**

A

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1 ATGTCCTCTGCGTTCATCGTCATGACGATGCTTCTGGGCTCTTCAGTGGTTTTTGCATTT
1 M S L A F I V M T M L L G S S V V F A F
61 GTCTTGCAGCCATCCGAGGAGGAACCTGCAGCCTCTGCTGACTCTCCTGTTTCCCATCAC
21 V L Q P S E E E P A A S A D S P V S H H
121 AGGTGCCAGGGTGGATCATTGCAGTCCATCAGGAAGGGTCTCCTCGGGGCTCCTCAACTTG
41 R C Q G G S L Q S I R K G L L G A L N L
181 CAGTTTGAGCCACGACTGCCTGCTGGTGGGCTGGACCATGTCAGAGAGCAATGGAGGACC
61 Q F E P R L P A G G L D H V R E Q W R T
241 ACCTACGGCACCATCGCTCACACGGCCAGGGACACTGCAGTCCAGCTGCCTCTGGCAAC
81 T Y G T I A H T A R D T A V P A A S G N
301 TCCGTGGCATCTGATGTTGAAACAGTACGAGCCTGAAGTGCTGTTCTATGGCCTCTGAG
101 S V A S D V G N S T S L K E C S M A S E
361 ATCTTCATGAAAGATCTGGGATGGGAAAGCTGGGTTATCGTTCCTGCCAGTGTACCATC
121 I F M K D L G W E S W V I V P A S V T I
421 GTTCAGTGTGCACTCTGCAACGCCGAAGGGAACACTGTGCAGTGTCCATCATCCCTTACC
141 V Q C A L C N A E G N T V Q C P S S L T
481 AATGTCCAGGCTGCAGACTCACAGGTGCCATGTTGTGAGCCCACTTCCCAGGAAACGGTG
161 N V Q A A D S Q V P C C Q P T S Q E T V
541 CATGTCTCTACGTGGATGAATCCGGCACCATCCACATTTCTCCATGCAGCTGACCCGC
181 H V L Y V D E S G T I H I S S M Q L T R
601 AGCTGCGGTGTCGGGCATGATAACCTCCAGCAGCCACCGGAGAGTAA
201 S C G C G H D N L Q Q P T G E *
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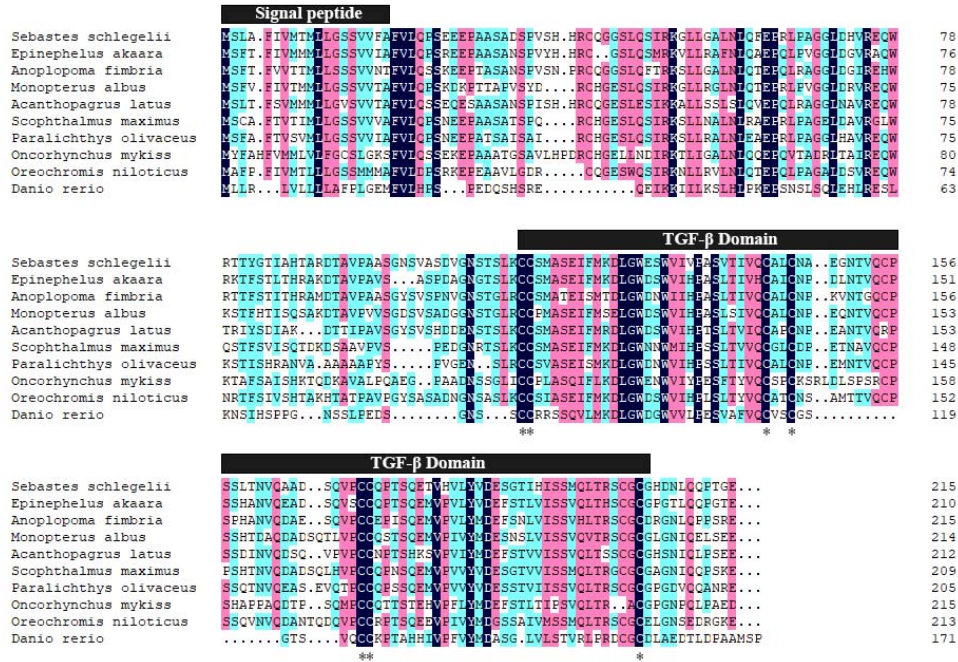
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237 Fig. 1. A: Nucleotide sequence of *Sgsdf* cDNA and deduced amino acid sequence.

238 The N-terminus signal peptide is underlined. The TGF- β region is shown in brownish

239 red. The numbers represent nucleotides and amino acids, respectively. B: The

240 domains of SsGsdF predicted by SMART program. The red box represents the signal
 241 peptide. The TGF- β region is shown in brownish red.

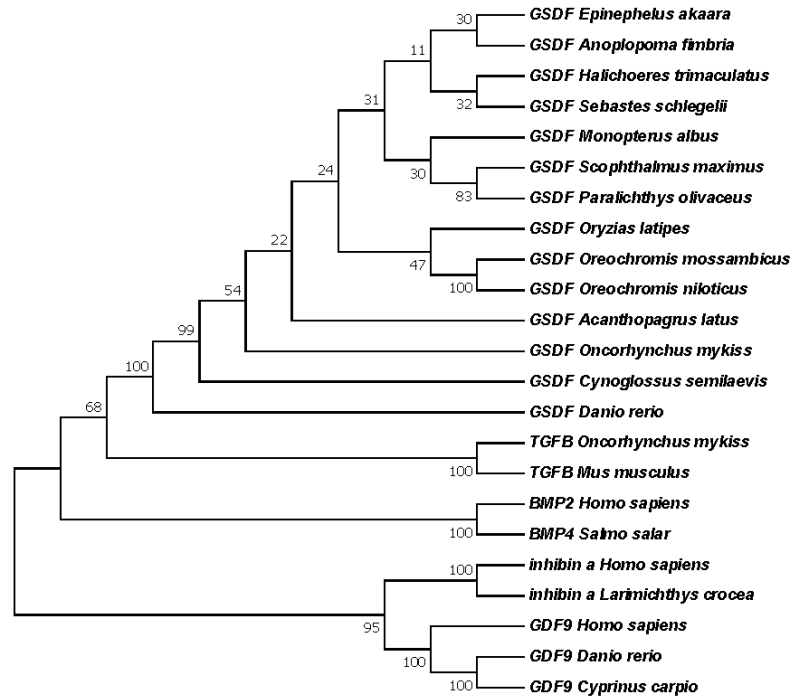


242 Fig. 2. Multiple alignment of deduced amino acid sequences of *gsdF* from *Sebastes*
 243 *schlegelii* and other fishes. The conserved cysteine residues are marked with asterisks.
 244
 245 Accession numbers are as follows: *Epinephelus akaara* (AIW52566.1), *Anoplopoma*
 246 *fimbria* (AGR33990.1), *Monopterus albus* (ALG62631.1), *Acanthopagrus latus*
 247 (AIW52571.1), *Scophthalmus maximus* (AJO67894.1), *Paralichthys olivaceus*
 248 (ARH56437.1), *Oncorhynchus mykiss* (NP_001118051.1), *Oreochromis niloticus*
 249 (BAJ78985.1), *Danio rerio* (ABZ01522.1).

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256 Fig. 3. Construction of phylogenetic tree with the protein sequences of *gsdf* and other
257 genes of TGF-β family. The phylogenetic tree was drawn by neighbor-joining method
258 replicates based on multiple sequence alignment by ClustalW and a bootstrap test
259 with 1000.

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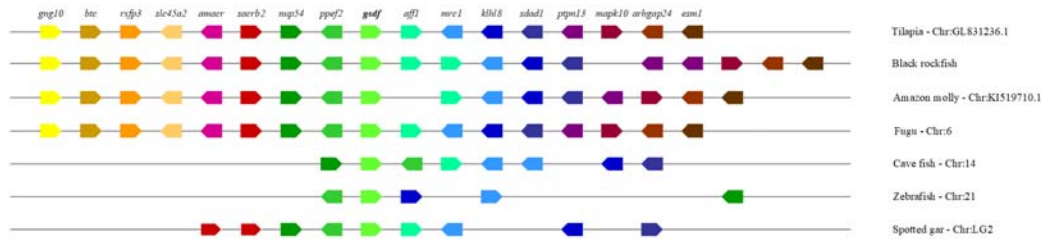
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270 Fig. 4. Chromosomal segments showing the synteny of *gsdf* in teleost. Different genes

271 are represented by different colored pentagons and gene order is determined according

272 to their relative positions in the chromosome or scaffold; the gene names are placed

273 on top of the pentagons. The direction of pentagons indicate the gene direction, the

274 vertical lines represent noncontiguous regions on the scaffold or chromosome.

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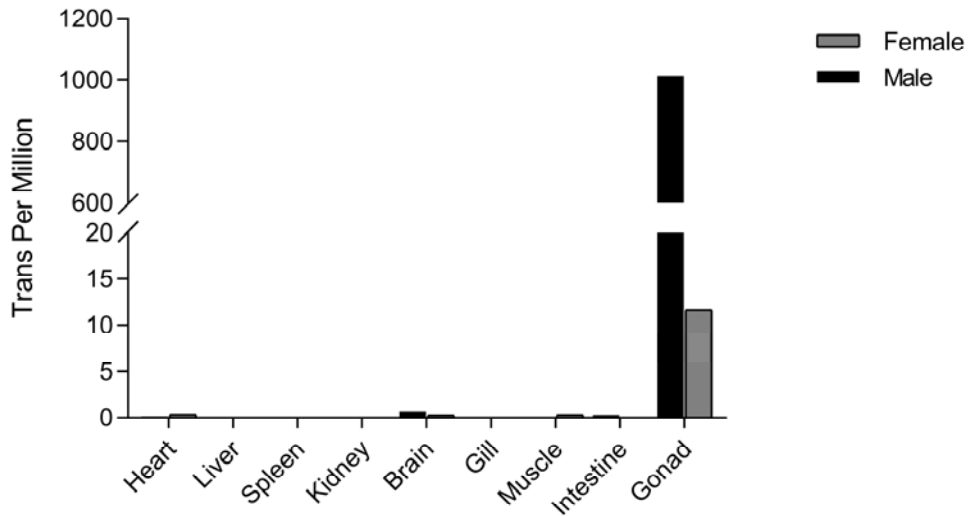
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288 Fig. 5. *Sgsdf* gene expression in different tissues. The expression level was measured
 289 with TPM scores. Numbers represent the mean value of TPM calculated with RNA-
 290 seq data of six individuals.

291

Table S1 Sequences information downloaded from NCBI

Gene name	Species	Accession number
<i>GSDF</i>	<i>Epinephelus akaara</i>	AIW52566.1
<i>GSDF</i>	<i>Anoplopoma fimbria</i>	AGR33990.1
<i>GSDF</i>	<i>Monopterus albus</i>	ALG62631.1
<i>GSDF</i>	<i>Halichoeres trimaculatus</i>	BAM75186.1
<i>GSDF</i>	<i>Acanthopagrus latus</i>	AIW52571.1
<i>GSDF</i>	<i>Scophthalmus maximus</i>	AJO67894.1
<i>GSDF</i>	<i>Oreochromis mossambicus</i>	ALO18792.1
<i>GSDF</i>	<i>Paralichthys olivaceus</i>	ARH56437.1
<i>GSDF</i>	<i>Cynoglossus semilaevis</i>	AYP19379.1
<i>GSDF</i>	<i>Oryzias latipes</i>	NP_001171213.1
<i>GSDF</i>	<i>Oncorhynchus mykiss</i>	NP_001118051.1
<i>GSDF</i>	<i>Oreochromis niloticus</i>	BAJ78985.1
<i>GSDF</i>	<i>Danio rerio</i>	ABZ01522.1
<i>BMP2</i>	<i>Homo sapiens</i>	NP_001191.1

<i>BMP4</i>	<i>Salmo salar</i>	NP_001133316.1
<i>inhibin a</i>	<i>Homo sapiens</i>	CAA01158.1
<i>inhibin a</i>	<i>Larimichthys crocea</i>	XP_027147478.1
<i>GDF9</i>	<i>Homo sapiens</i>	EAW62309.1
<i>GDF9</i>	<i>Danio rerio</i>	AAV91155.1
<i>GDF9</i>	<i>Cyprinus carpio</i>	AOW71519.1

UNDER PEER REVIEW