1 *Short Research Article*

The Establishment of a Fibroblastic Cell Line From Caudal Tissue of *Poecilia reticulata*, Peters 1859

ABSTRACT

Establishment of cell lines from different tissues of different teleost fishes has remarkably increased. Development of fish cell lines has significantly contributed to recent advances in many research areas. *Poecilia reticulata* also known as guppy is a teleost fish which is widely used for ecotoxicological research. In this study to isolate fibroblast cell from caudal tissue of *P. reticulata* and to develop a cell line.For this purpose caudal tissue samples were collected from ten male *P.reticulata* under aseptic conditions. Primary culture was performed with tissue explant technique. Primary cells fed with L15 culture medium supplemented with FBS. To investigate the effects of different incubation temperature on proliferation rate of cells, seven different incubation temperatures (15°C, 20°C, 25°C, 28°C, 30°C, 32°C, 37°C) were assessed. Same experimental design was used for the effects of different FBS concentration (5%, 10%, 15%, and 20%) on proliferation rate of cells. Optimum growth rate was observed at 28°C temperature and 10% FBS concentration. Cells were subcultured succesfuly more than 60 times and this resulted in development of a cell line named as PSF. Cultured cells were also succesfuly cryopreserved. The revival rate of PSF cell line was up to %85-90 and this result could be admitted as a good when compared to other fish cell lines.

Keywords: Poecilia reticulata, cell culture, caudal fibroblast, cell line

1. INTRODUCTION

In the recent years, establishment of cell lines from different organs or tissues of teleost fishes has remarkably increased. First cell line was reported in 1952 by Wolf and Guimby [1]. Since that time, about 238 cell line have been established from teleost around the World [2]. The development of fish cell culture and cell lines has significantly contributed to recent advances in toxicology [3, 4], virology [5] and also cytogenetics [6], biomedical research [7], biotechnology and aquaculture [8], endocrinology [9], fish immunology [10, 11], ecotoxicology [12, 13, 14], disease control [15] and radiation biology [16]. Investigating of environmental pollution and effects of xenobiotics on living organisms, in vitro techniques and cell culture methods is widely used for replacement of animals [12, 17]. Many cell lines especially isolated from gonads, liver, gills and total embryo tissue of fishes has been used for investigate the effects of various chemicals in cellular level. Fibroblast cells isolated from caudal biopsies of fishes can also be used for that purpose [12].

Poecilia reticulata, also called guppy or milionfish is a world wide distrubuted tropical fish. Because of its smal size, easy acclimation of laboratory conditions, easy maintenance and short live cycle and reproduction, it has become widely used laboratory model for ecotoxicological research [17]. A cell line named as GFT was isolated from fully formed unborn embryos of a female guppy [18], but there is no other cell line has established according to literature [18]. When compared other cultured cells, fibroblast cells can be sampled more easily and provide material for rapid and early genetic screening. Also fibroblast cells yield high quality chromosomes in fish species [19]. Caudal fin is one of the main source of fibroblast tissue and methodologically easy way to isolate fibroblastic cells.The aim of this study was to develop a cell line from caudal fin tissue of guppy and optimize culture conditions.

2. MATERIAL AND METHODS

33 **2.1 Tissue Explant:** All experiments have been examined and approved by Animal Experiment
34 Ethics Committee of Ege University. Healthy juveniles (ten male) of *P.reticulata* were obtained local
35 commercial supply and maintained at the Ege University Science Faculty Biology Department Zoology
36 Section, Ecotoxicology laboratory. Fishes were starved for a day and allowed to swim in well-aerated
37 sterile water before the collecting of fin tissue. The specimens were anaesthetized with keeping them on
38 +4 °C cold water for 5-7 min. Then surface sterilized by wiped with %70 alcohol.

39 Primary cell cultures were initiated by collecting caudal fin tissues from the fishes under aseptic
40 conditions. The tissue samples were transferred into phosphate buffered saline supplemented with 10
41 μg/ml Gentamycin (Gibco). The samples were minced with sterile scissors at room temperature and
42 washed three times with washing buffer.

43 The minced tissue fragments were seeded into 25 cm^2 tissue culture flasks [TPP, Switzerland] in
44 nearly 1 ml of L15 culture media supplemented with %10 FBS. The explants allowed to attached to the
45 surface of the flask in an incubator at 28 ºC for 4 h. Care was taken to avoid over drying to the tissue.
46 After observing tissue adherence, the explants were fed with L15 culture media supplemented with %10
47 FBS.

48 **2.2 Subculture:** For proliferation of cells and morphological details, the flasks were observed
49 daily with using inverted microscope (Olympus Tokyo japan). After cells reach %95 confluency they were
50 trypsinized using a trypsin EDTA solution (trypsin 0.25%, EDTA 0.02%) in phosphate buffered saline. The
51 subcultured cells were grown in fresh L15 with %10 FBS.

52 **2.3 Growth Studies:** For assessment of growth characteristics of the cell line in L15 media,
53 cells were incubated different incubation temperatures (15°C, 20°C, 25°C, 28°C, 30°C, 32°C, 37°C) for 7
54 days. On the consecutive days, three flasks from different temperatures at which they were incubated
55 were withdrawn, the cell lines trypsinized and cell counting performed using a hemocytometer. Same
56 procedure were performed for the effects of various concentrations of FBS (5%, 10%, 15%, and 20%) on
57 cell growth at 28 ºC for 7 days.

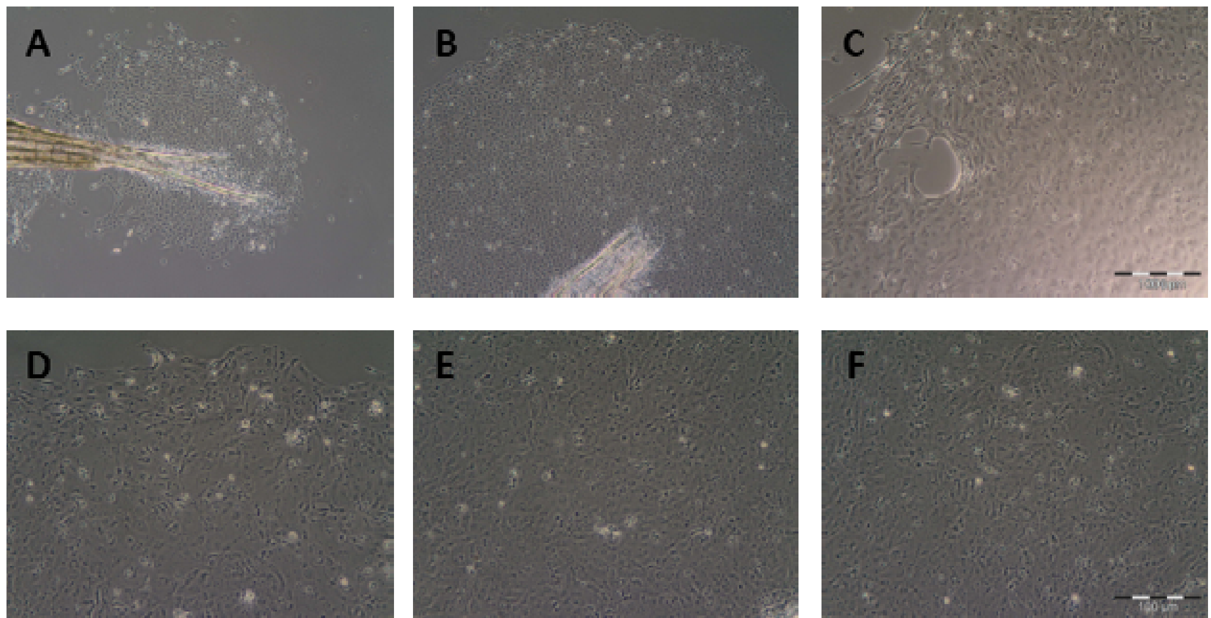
58 **2.4 Cryopreservation:** To observe the ability of cells to survive in liquid nitrogen and stability
59 of them were assessed in freezing medium using the method that described by Freshney [20].
60 Logarithmically growing cells were harvested and re-suspended at $3x10^6$ to $4x10^6$ cells ml^{-1} densities. The
61 cell suspensions were carefully mixed with equal volume of recovery medium (Invitrogen) which is
62 suitable complete cryopreservation medium for freezing cells (Invitrogen) at $1x10^6$ cells mL^{-1} according to
63 manufacturer instructions. The recovery medium could increase cell viabilities average of 25% in
64 cryopreservation both adherent and suspension cell lines and it is an optimized fully supplemented
65 formulation which avoids the messy mixing of DMSO. Cell suspensions were aliquoted (1ml) and
66 distributed into 1,5 sterile cryovials (Nunc) held at 4°C for 2 h, -20°C for 2h and -70°C overnight then
67 transferred into liquid nitrogen (-196°C). The frozen cells were thawed after 6 months of post storage at
68 37°C water bath. Freezing medium was removed by centrifugation and cells were suspended in L15 with
69 10% FBS. Cell viability measured by trypan blue staining and number of cells was counted using
70 hemocytometer. Cells were seeded into 25cm^2 tissue culture flasks for further subculturing.

71 **3. RESULTS AND DISCUSSION**

72 The cell line named as PSF from caudal fin tissue of *P. reticulata* was established by explant
73 technique. The explant technique has many advantages such as speed, easy maintenance of cellular
74 interactions and no enzymatic damage of cell surface proteins over the trypsinization method [21].

75 For morphological and other examinations, cells were observed by inverted microscope every day
76 during subsequent passages (figure 1). Culture medium was changed every 3 day. Initially, fibroblastic
77 and epithelial cells were found at the beginning of the culture, but after 4th passage, fibroblastic cells
78 dominated over the epithelial cells (figure 1). PSF cells were predominantly observed as fibroblastic cells

79 at 10th passages (figure 1). During early passages, the heterogeneity nature of cells during early
80 passages was also reported by other researchers [22, 23, 24]. The predomination of fibroblastic cells
81 over epithelial cells in cell cultures from fin tissue of fishes also has been reported before [25, 26, 27]. A
82 fibroblast like cell line [LJH-2] was developed from *Lateolabrax japonicus* [28]. In contrast cells isolated
83 from heart tissue of sea perch, have been reported to be epithelial morphology with no change during
84 subsequent culture [29]. Cells started to spread after 4-5 days of explant preparation from fins. Confluent
85 monolayer cells were observed 10-12 days of culture (figure 1) Cells were subcultured in L15 with %10
86 FBS at 5-7 day of interval and split at ratio of 1:2. Cells were subcultured more than 60 times regularly
87 and this resulted in development of a cell line named as PSF.

88 

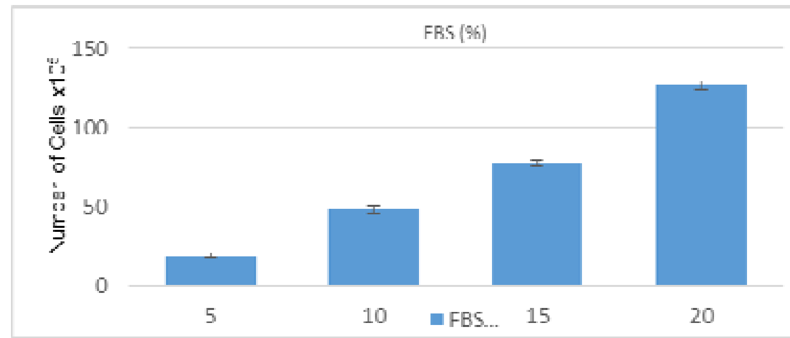
89 Figure 1: microscopic observation of cells, A: 3th day of culture (spreading cells from fin tissue), B: 5th day of culture
90 (proliferating and increasing of cells) , C: 10 day of culture (confluency of cells), D: 4th passage of cells (dominating of fibroblast
91 cells), E: 10th passage of cells (fibroblast cells are become stabilized), F: 60th day of culture (dominated and stabilized fibroblast
92 cells).

93 The effects of different FBS concentration on proliferation rate have been examined and shown in
94 fig 2 . For this aim, four different (5%, 10%,15%,20%) FBS concentration have been examined.
95 Relatively, growth rate of cells increased as the FBS portion increased from %5 to %20 at 28 °C
96 incubation temperature. However, growth rate was observed as minimum at %5 concentration of FBS
97 and maximum at %10 and 15 FBS. Comparatively fish cell lines have been exhibited good growth at %10
98 FBS but maximum growth at %15 and %20 FBS concentrations [30, 31, 28].

99

100

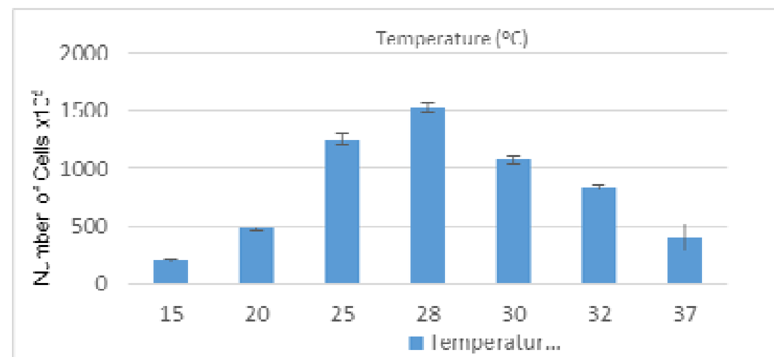
101



102 Fig. 2. proliferation of cells on different FBS concentration

103 For observation of different growth patterns of PSF cell line at different incubation temperatures
104 between 15°C and 37°C was examined and shown in Fig. 2.1 . The minimum growth rate was observed
105 at 15 °C incubation temperature. The monolayer detachment was observed 37 °C incubated cells.
106 However optimum culture temperature was found to be 28 °C. This temperature also reported for other
107 fish cell lines in the literature before (22, 32, 33, 34, 35, 36, 24).

108



109 Fig. 2.1: proliferation of cells on different incubation temperatures

110 As described before, ideal growth temperature for cultured fish cells is a few degrees above
111 environmental living temperature of fish. [38], because of selection or adaptation of cells to growth of
112 higher than normal temperature [39]. For tong et al [40], temperatures between 35-37°C has been found
113 lethal fort o many fish cells. As seen in the literature, fish cells could be growth at over wide temperature
114 range. This could be an advantage for isolating both warm water and coldwater fish viruses [41].

115 The cells that grow in medium containing %20 FBS has showed higher proliferation rate than
116 cells in medium containing %5-15 FBS (Fig. 2). The growth rate of the cells increased paralel with FBS
117 concentration from %5 to %20. Nevertheless %10-15 concentration of FBS also provided good growth.
118 So it would be an advantage to use %10 FBS to maintain the cell line because of low cost. In the
119 literature, relatively good growth of the fish cell observed at %10 FBS but maximum growth also observet
120 at the concentration of %15 to %20 (30,27, 28).

121 Protection of cell lines from genetic change and avoid aging and transformation, cryopreservation
122 is needed in long term storage [20]. The PSF cell line freezed in liquid nitrogen and storage during 6
123 months. When cells were thawed, they showed %85-90 viability and continued to grow and became
124 confluency within 7 days. The revival rate of the PSF cell line was up to %85-90. This result could be
125 admited as a good when compared to that of other fish cell lines reported by Parameswaran et al. [42]
126 who could revive SISE cell succesfully after cryopreservation with the survival rate of %70-80. The GFM
127 and GSFB cell lines showed %93 and %92 survival rate after cryopreservation according to Roughee et

128 al [43]. The cell line named as MFF-1 from mandarin fish, *Siniperca chuatsi* revived %80-90 after
129 cryopreservation [44]. Cells were cryopreserved and succesfully revived with the survival rates of %75,
130 %70 and %72 for RHi RF and RSB cell lines [2]. Swaminthan et al. [33] succesfully cryopreserved and
131 revived with the survival rate of %70. Cell line named as PSF succesfully cryopreserved and revived with
132 the survival rate of %75 by Lakra and Goswami [31].

133 **4. CONCLUSION**

134 In conclusion, the present study results in the development of cell line PSF from the caudal fin of
135 *P. reticulata*. The development of cell line PSF from *P. reticulata* has not been reported before. Therefore,
136 development of a cell line form tissues of guppy (*P.reticulata*) could open new horizons of in vitro
137 research in fish genetics, conservation and fish biotechnology. Also it could further be used for developing
138 cellular models for toxicological, ecotoxicological and genotoxicological studies to replace whole animals.

139 **REFERENCES**

140 1. Wolf K, Quimby MC. Established Eurythermic Line of Fish Cells in vitro. Science.
141 1962;135[3508]:1065-6
142 2. Lakra WS, Swaminathan TR, Rathore G, Goswami M, Yadav K, Kapoor S. Development and
143 characterization of three new diploid cell lines from Labeo rohita [Ham.]. Biotechnology Progress.
144 2010;26[4]:1008-1013.
145 3. Babich H, and Borenfreund E. Cytotoxıcıty and Genotoxıcıty Assays with Cultured Fısh Cells: A
146 Revıew. Toxicology in vitro. 1991;5[1]: 91-100.
147 4. Segner H. Fish Cell Lines as a Tool in Aquatic Toxicology. Experientia Supplementum.
148 1998;86:1– 38;
149 5. Wolf K. 1988. Fish Viruses and Fish Viral Diseases. Cornell University Press, New York.
150 6. Blaxhall PC. Lymphocyte culture for chromosome preparation. Journal of Fish Biology.
151 1983;22[3]:279–282.
152 7. Hightower L, Renfro E. Recent applications of fish cell culture to biomedical research. Journal of
153 Experimental Zoology. 1988;248:290-302.
154 8. Bols NC. Biotechnology and aquaculture: the role of cell cultures. Biotechnology Advantages.
155 1991;9:31–49.
156 9. Bols NC, Lee LEJ. Technology and uses of cell cultures from the tissues and organs of bony fish.
157 Cytotechnology. 1991;6:163–187.
158 10. Clem LW, Bly JE, Wilson M, Chinchar VG, Stuge T, Barker K, Luft C, Rycyzyn M, Hogan RJ, Van
159 Lopik T, Miller NW. Fish immunology: the utility of immortalized lymphoid cells—a mini review.
160 Veterinary Immunology and Immunopathology. 1996;54:137–144.
161 11. Bols NC, Brubacher JL, Ganassin RC, Lee LEJ. Ecotoxicology and innate immunity in fish. Dev.
162 Comp. Immunol. 2001;25:853–873.
163 12. Fent K. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome
164 P4501A induction potential and estrogenic activity of chemicals and environmental samples.
165 Toxicology in vitro. 2001:15;477–488.
166 13. Castano A, Bols N, Braunbeck T, Dierickx P, Halder MB, Kawahara K, Lee LEJ, Mothersill C, Part
167 P, Repetto G, Sintes JR, Rufli H, Smith R, Wood C, Segner H. The use of fish cells in
168 ecotoxicology. ATLA. 2003:31;317–351.
169 14. Schirmer K. Proposal to improve vertebrate cell cultures to establish them as substitutes for the
170 regulatory testing of chemicals and effluents using fish. Toxicology. 2006: 224[3];163–183.
171 15. Villena AJ, Applications and needs of fish and shellfish cell culture for disease control in
172 aquaculture. Reviews in Fish Biology and Fisheries. 2003:13[1];111-140.
173 16. Ryan LA, Seymour CB, O'Neill-Mehlenbacher A, Mothersill CE. Radiation-induced adaptive
174 response in fish cell lines. Journal of Environmental Radioactivity. 2008:99[4]; 739–747.
175 17. Fent K. Permanent Fish Cell Cultures as Important Tools in Ecotoxicology. ALTEX. 2007:
176 24;Special Issue.

177 18. Li MF, Marrayatt V, Annand C, Odense P. Fish cell culture: two newly developed cell lines from
178 Atlantic sturgeon [Acipenser oxyrhynchus] and guppy [Poecillia reticulata]. Canadian Journal of
179 Zoology. 1984:63;2867-2874.
180 19. Amemiya CT, Bickham JW, Gold JR. A Cell Culture Technique for Chromosome Preparation in
181 Cyprinid Fishes. Copeia. 1984: 232–235.
182 20. Freshney RI, Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications.
183 6th ed. John Wiley & Sons, Inc., Hoboken, NJ, USA; 2010
184 21. Avella M, Berhaut J, Payan P. Primary cuture of gill epithelial cells from the sea bass
185 Dicentrarchus labrax. In Vitro Cellular and Developmental Biology. 1994;30[A]:41–49.
186 22. Parameswaran V, Shukla R, Bhonde RR, Hameed ASS. Splenic cell line from sea bass, Lates
187 calcarifer: establishment and characterization. Aquaculture 2006:261[B];43–53.
188 23. Lakra WS, Goswami M, Swaminathan TR, Rathore G. Development and characterization of two
189 new cell lines from common carp, Cyprinus carpio [Linn]. Biological Research. 2010:43;385–392.
190 24. Kapoor S, Bhatt JP, Lakra WS. Development and characterization of a new cell line CF from
191 caudal fin of knifefish, Chitala chitala [Hamilton-Buchanan]. In Vitro Cellular & Developmental
192 Biology. 2013;49[9]:728–733.
193 25. Bejar J, Borrego JJ, Alvarez MC. A continuous cell line from the cultured marine fish gilt-head sea
194 bream [Sparus aurata]. Aquaculture. 1997;150:143–153.
195 26. Lai YS, John JAC, Lin CH, Guo IC, Chen SC, Fang F, Lin CH, Chang CY. Establishment of cell
196 lines from a tropical grouper, Epinephelus awoara [Temminck and Schlegel], and their
197 susceptibility to grouper irido and nodaviruses. Journal of Fish Diseases. 2003;26: 31–42
198 27. Lakra WS, Bhonde RR, Sivakumar N, Ayyappan S. A new fibroblast like cell line from the fry of
199 golden mahseer Tor putitora [Ham]. Aquaculture. 2006;253[A]:238–243.
200 28. Ye HQ, Chen SL, Sha ZX, Xu MY. Development and characterization of cell lines from heart,
201 liver, spleen and head kidney of sea perch Lateolabrax japonicus. Journal of Fish Biology,
202 2006;69:115–126.
203 29. Tong SL, Miao HZ, Li H. Three new continuous fish cell lines of SPH, SPS and RSBF derived
204 from sea perch [Lateolabrax japaonicus] and red sea bream [Pagrosomus major]. Aquaculture.
205 1998;169:143–151.
206 30. Hameed ASS, Parameswaran V, Shukla R, Bright Singh IS, Thirunavukkarassu AR, Bhonde RR.
207 Establishment and characterization of India's first marine fish cell line [SISK] from the kidney of
208 sea bass [Lates calcarifer]. Aquaculture. 2006;257:92–103.
209 31. Lakra WS, Goswami M. Development and characterization of a continuous cell line PSCF from
210 Puntius sophor. Journal of Fish Biology. 2011;78[4]:987–1001.
211 32. Ahmed IVP, Chandra V, Sudhakaran R, Rajesh Kumar S, Sarathi M, Sarath Babu V, Ramesh B,
212 Hameed ASS. Development and characterization of cell lines derived from Rohu, Labeo rohita
213 [Hamilton], and Catla, Catla catla [Hamilton]. Journal of Fish Diseases. 2009:32;211–218.
214 33. Swaminathan TR, Lakra WS, Gopalakrishnan A, Basheer VS, Khushwaha B, Sajeela KA.
215 Development and characterization of a new epithelial cell line PSF from caudal fin of green
216 chromide, Etroplus suratensis [Bloch, 1790]. In Vitro Cellular and Developmental Biology-Animal.
217 2010;46[8]:647–656.
218 34. Swaminathan TR, Lakra WS, Gopalakrishnan A, Basheer VS, Khushwaha B, Sajeela KA.
219 Development and characterization of a fibroblastic-like cell line from caudal fin of the red-line
220 torpedo, Puntius denisonii [Day] [Teleostei:Cyprinidae]. Aquatic Research 2011;43[4]:498–508.
221 35. Babu VS, Nambi KSN, Chandra V, Ahmed VPI, Bhonde R, Hameed ASS. Establishment and
222 characterization of a fin cell line from Indian walking catfish, Clarias batrachus [L.]. Journal of Fish
223 Diseases. 2011;34[5]:355–64.
224 36. Lakra WS, Goswami M. Development and characterization of a continuous cell line PSCF from
225 Puntius sophor. Journal of Fish Biology. 2011:78[4];987–1001.
226 37. Lakra WS, Sivakumar N, Goswami M, Bhonde RR. Development of two cell culture systems from
227 Asian seabass Lates calcarifer [Bloch]. Aquaculture Research 2006;37:18–24.
228 38. Alvarez MC, Otis J, Amores A, Guise K. Short-term cell culture technique for obtaining
229 chromosomes in marine and fresh water. Journal of Fish Biology. 1991;39:817–824.
230 39. Wolf K, Ahne W. Fish cell culture. In: Maramorosch K. [ed] Advances in cell culture, vol. 2.
231 Academic Press, New York, 1982. pp 305–328; 1982.

232 40. Tong SL, Lee H, Miao HZ. The establishment and partial characterization of a continuous fish cell
233 line FG-9307 from the gill of flounder, Paralichthys olivaceus. Aquaculture. 1997;156:327–333.
234 41. Nicholson BL, Danner DJ, Wu JL. Three new continuous cell lines from marine fishes of Asia. In
235 Vitro Cellular and Developmental Biology. 1987;23:199–204.
236 42. Parameswaran V, Shukla R, Bhonde RR, Hameed ASS. Establishment of embryonic cell line
237 from sea bass [Lates calcarifer] for virus isolation. Journal of Virological Methods. 2006;137:309–
238 316.
239 43. Rougee L, Ostrander GK, Richmond RH, Yuanan L. Establishment, characterization and viral
240 susceptibility of two cell lines derived from goldfish Carassius auratus muscle and swim bladder.
241 Diseases of Aquatic Organisms. 2007;77:127–135.
242 44. Dong C, Weng S, Shi X, Shi N, He J. Development of a Mandarin fish, Siniperca chuatsi fry cell
243 line suitable for the study of infectious spleen and kidney necrosis virus [ISKNV]. Virus Research.
244 2008;135:273–281.