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银鲫不同克隆系疱疹病毒抗性差异及全基因组关联分析

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摘要: 为了探究银鲫不同克隆系在鲫疱疹病毒抗性上的差异及鉴定潜在抗病相关基因, 研究首先构建了7个雌核生殖克隆系, 并对其母本进行了微卫星标记鉴定。这7个克隆系包括方正银鲫克隆系1 (FZ1)、方正银鲫克隆系2 (FZ2)、方正银鲫克隆系3 (FZ3)、鄱阳银鲫克隆系(PY)和彭泽银鲫克隆系(PZ), 以及作为对照的高抗品系H系和易感品种“中科3号”A⁺系。随后, 对这些银鲫克隆系进行鲫疱疹病毒人工感染试验, 存活率由高到低依次为H (100%) > FZ2 (95%) > PY (40%) > FZ1 (20%) = FZ3 (20%) = PZ (20%) > A⁺ (10%)。人工感染后48h, 高抗克隆系FZ2和H的肝脏细胞排列更紧密, 且鲫疱疹病毒基因表达水平更低, 暗示其肝脏病毒载量低; 而易感克隆系FZ1、FZ3、PZ和A⁺的肝脏细胞更肿大, 且鲫疱疹病毒基因表达水平更高, 暗示肝脏病毒载量高。此外, 研究对每个克隆系的10尾子代进行了基因组重测序, 鉴定到35278852个SNP (Single Nucleotide Polymorphisms)位点。基于全基因组关联分析, 研究筛选到63个与抗病性状相关的基因组区域, 这些区域总共含有213个注释基因。通过与转录组数据联合分析及荧光定量PCR验证, 最终筛选到11个潜在抗病相关基因。研究不仅鉴定出具有较高鲫疱疹病毒抗性的优良种质, 还筛选出银鲫潜在抗病相关基因, 为银鲫抗病育种工作奠定了基础。

关键词: 雌核生殖; 克隆系; 鲫疱疹病毒; 抗病育种; 银鲫

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鲫复合种(*Carassius species complex*)包含双二倍体鲫(*C. auratus*)和双三倍体银鲫(*C. gibelio*)^[1], 隶属于硬骨鱼纲(Osteichthyes), 鲤形目(Cypriniformes), 鲤科(Cyprinidae), 鲫属(*Carassius*), 广泛分布于亚欧大陆及其临近岛屿的淡水水系^[2]。双二倍体鲫含有两套二倍体基因组(AABB, $n=100$)^[3], 行有性生殖,

具有XX/XY性别决定系统^[4]; 双三倍体银鲫包含两套三倍体基因组(AAABBB, $n\approx 150$)^[3], 行单性雌核生殖, 具有超数染色体性别决定系统^[5]。鲫复合种具有多面性, 包含多种倍性、多重生殖方式和不同性别决定系统, 这些特征使其成为演化发育生物学研究的理想系统^[6, 7]。同时, 鲫复合种也是我国重

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要的大宗淡水养殖鱼类。随着双三倍体银鲫天然雌核生殖方式的发现及其在育种上的应用,改变了我国银鲫养殖状态及产业发展,全国鲫总产量也从1983年的4.8万吨增至2023年的284万吨^[8]。然而,目前银鲫养殖过程中病害频发,尤其是鲫疱疹病毒(*Carassius auratus herpesvirus*, CaHV)引发的出血病,给银鲫养殖产业造成了重大损失^[9]。

双三倍体银鲫进行天然雌核生殖,产生不减数的卵子,卵子利用同水域有性生殖物种的精子刺激其胚胎发育,产生遗传信息与母本一致的全雌子代^[10]。因此,雌核生殖系也称为克隆系,克隆系子代个体间几乎没有遗传差异。正因为银鲫雌核生殖子代遗传信息一致,不能在雌核生殖系内部去筛选优异种质,但可以对不同雌核生殖克隆系进行比较,筛选具有优异性状的克隆系。在实验室前期系统的研究工作中,利用全国搜集的野生优势个体,构建了多个银鲫克隆系,如A、B、C、D、E系等^[11]。在A和D系的基础上,利用雄核生殖培育了异育银鲫“中科3号”(A⁺系)新品种^[12];在E系的基础上,利用冷休克技术将团头鲂的DNA片段整入E系基因组中,培育了异育银鲫“中科5号”(F系)新品种^[13]。这些优异新品种均是以野生优异种质为基础,利用先进水产生物技术培育而成^[14]。因此,进一步筛选和鉴定我国地理种群中的优异种质,对于银鲫遗传育种具有重要意义^[15,16]。

本研究搜集了方正银鲫、鄱阳银鲫和彭泽鲫等地理种群,并在其中挑选具有体型优势的个体构建了5个雌核生殖克隆系:包括方正银鲫克隆系1(FZ1)、方正银鲫克隆系2(FZ2)、方正银鲫克隆系3(FZ3)、鄱阳银鲫克隆系(PY)和彭泽鲫克隆系(PZ)。本文聚焦鲫疱疹病毒抗性,以2个抗性已知的克隆系为对照:高抗品系H系和易感品种异育银鲫“中科3号”(A⁺系)。通过人工感染后的存活率、肝脏细胞形态及病毒基因表达水平分析,鉴定到具有较强鲫疱疹病毒抗性的方正银鲫克隆系2。此外,利用基因组重测序和全基因组关联分析,筛选到63个与抗病性状相关的基因组区域。联合转录组分析和荧光定量PCR验证,最终筛选到11个潜在抗病相关基因。这些结果为银鲫抗病遗传育种工作奠定了基础。

1 材料与方法

1.1 实验材料

本研究收集了方正银鲫、鄱阳银鲫和彭泽鲫等地理种群,并保存在国家水生生物种质资源库(National Aquatic Biological Resource Center, NABRC),

中国武汉。H系银鲫、A⁺系银鲫及雌核生殖父本兴国红鲤由国家水生生物种质资源库提供,鲫疱疹病毒(CaHV)来自中国科学院水生生物研究所,中国武汉。

1.2 银鲫雌核生殖克隆系构建

在银鲫繁殖季节,对性腺发育良好的7尾母本(方正银鲫3尾、鄱阳银鲫1尾、彭泽鲫1尾、H系1尾、A⁺系1尾)进行腹腔注射催产素(地欧酮、丙酮干鲤垂体、人绒毛膜促性腺激素和促黄体素释放激素A₂按比例溶于0.75%的生理盐水)进行人工催产^[17]。注射8h左右,雌鱼产卵,将卵子挤在玻璃皿中,将鲤精液挤在卵子上,用干净的羽毛轻轻搅拌均匀后,均匀地铺在网片上,并放置于网箱中,(21±1)℃下孵化。出膜后的小鱼苗用丰年虫喂养^[18]。不同克隆系的鱼苗养殖在同一水体的大小相同的网箱中。

1.3 微卫星鉴定

剪取适量大小的尾鳍,用蒸馏水冲洗干净,滤纸吸干水分,放入1.5 mL离心管。加入500 μL核酸裂解液(Promega, 北京)和10 μL蛋白酶K (Sigma), 65℃水浴30min。待消化完全后使用异丙醇沉淀法提取基因组DNA,最后加入50 μL 灭菌水与DNA充分混匀^[19],使用NanoDrop测浓度,将DNA浓度稀释至50 ng/μL, 4℃保存备用。PCR体系为25 μL: 2×Es Taq Master Mix (Dye; 康为, 北京)12.5 μL、Primer F和Primer R各1 μL、DNA 1 μL、ddH₂O 9.5 μL。7对微卫星引物序列来源于已经发表的文章: HLJYJ034、HLJYJ090^[20]、HLJYJ029^[21]、YJ0004、YJ0010、YJ0033^[22]和GCE401^[23],引物由上海生工生物技术有限公司合成。PCR反应条件: 95℃预变性3min; 95℃变性30s, 60℃退火30s, 72℃延伸40s, 35个循环; 72℃终延伸5min, 12℃ 2min。PCR产物用10%聚丙烯酰胺凝胶(PAGE)进行分离, PAGE胶配置: H₂O 13 mL、5×Tris-硼酸(TBE)电泳缓冲液(吉至生化科技) 5 mL、30%亚甲基双丙烯酰胺溶液Acr (伊势久生物科技, 江苏)10 mL、10%过硫酸铵Aps (酷来搏科技, 中国) 400 μL、四甲基乙二胺TEMED(伊势久生物科技) 10 μL。待胶凝固后,每个孔加入PCR产物3 μL, Marker是pUC18 DNA/MspI (庄盟生物)。电压230 V, 电流110 A, 电泳时间为3h。在凝胶电泳结束后,使用核酸染料GelRed (Biosharp)与水的混合物进行染色5min, 扫胶仪(BioRad)扫胶^[24]。

1.4 人工感染试验

不同克隆系鱼苗在网箱中以相同密度养殖到4月龄,每个克隆系取30尾大小均匀的个体进行人

工感染试验。使用微量注射器吸取10 μL 病毒液体,其中病毒滴度为 5×10^7 TCID₅₀/mL, TCID₅₀ (Tissue Culture Infectious Dose 50%): 半数细胞培养物感染剂量。注射前摆至腹部向上体位,自泄殖孔进针,针尖探入腹腔,缓缓推入病毒悬液。攻毒后分别养殖在10 L水体中,观察并统计存活率,出现死鱼后将死鱼捞走。人工感染试验进行2次。此外,攻毒48h后,取各5尾个体的肝脏和头肾组织,用于RNA提取和切片。用于RNA提取的样品放入液氮速冻,研磨成粉末状后加入TRIzol (Invitrogen, 美国)裂解。

1.5 苏木精-伊红染色法(Hematoxylin-eosin staining, HE)染色

用于切片的肝脏样品放入4%多聚甲醛固定液(Servicebio, 武汉)4℃固定过夜,将固定好的样品在不同浓度乙醇系列(70%、80%、90%、100%)中脱水处理,用柠檬烯(Lemosol, Wako, 日本)进行透明化处理,然后进行石蜡包埋及切片,切片厚度4 μm ,苏木精-伊红染色过程如文献[25]所述。

1.6 实时荧光定量PCR (Relative quantitative real-time PCR, qPCR)

qPCR采用SYBR Green染料法,根据操作说明进行基因表达量的检测。总RNA采用SV总RNA分离系统(Promega, 美国),按照说明书的步骤进行分离。使用NanoDrop2000紫外分光光度计(Thermo Fisher, 美国)测定RNA的浓度,使用琼脂糖凝胶电泳检测总RNA的质量。qPCR反应体系为20 μL ,包括10 μL 的iTaq™ Universal SYBR® Green Super mix (TaKaRa, 日本),0.5 μL 的上游引物和0.5 μL 的下游引物,1 μL 的模板和8 μL 的灭菌水。反应程序:95℃预变性30s;95℃变性30s,60℃退火30s,72℃延伸20s,40个循环。在扩增结束后,进行熔解曲线分析(从65℃升温至95℃,每0.5℃读取1次荧光信号,绘制熔解曲线)。每个反应进行3次重复,以 β -actin作为内参,采用 $2^{-\Delta\Delta C_t}$ 计算获得基因的相对表达量[26]。

1.7 全基因组重测序

每个克隆系取10尾子代的肌肉组织进行全基因组重测序,所有提取的DNA经过质量检测,均符合测序要求。使用300—400 bp全基因组测序(WGS, Whole Genome Sequencing)文库方法构建基因组DNA文库,使用DNBSEQ-T7平台PE150进行测序[27, 28]。基于序列比对结果,利用picard软件去除PCR重复,然后剔除比对质量低于20的数据,最后采用GATK (Genome Analysis Toolkit)软件进行群体检测,获得原始单核苷酸多态性SNP变异检测信

息[28]。为获得高质量的变异检测结果,本文利用GATK软件采用如下参数进行质量过滤,SNP过滤参数:QD < 2.0 || FS > 60.0 || MQ < 40.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0[29],其中,QD是变异可信度与测序深度的比值,FS衡量链偏向程度,MQ是比对质量的中位数,MQRankSum和ReadPosRankSum分别衡量变异位点的比对质量和位置分布情况。利用VCFtools软件[30, 31]进行群体过滤,只保留次等位基因频率(Minor Allele Frequency, MAF) > 0.05,位点缺失率(Missing Rate) < 25%,最小平均深度(Minimum mean Depth, min-meanDP)为5的二等位基因位点。参数设置:--maf 0.05 --min-alleles 2 --max-alleles 2 --min-meanDP 5 --max-missing 0.75[32]。最终获得高质量的SNP数据用于后续分析。

1.8 全基因组关联研究

利用VCFtools软件[30],以100 kb为窗口,10 kb为步长,进行群体分化分析。之后分别统计组内碱基频率,用于组间欧式距离计算,然后以100 kb为窗口,10 kb为步长,计算组间窗口欧式距离并进行乘方处理。最后分别统计组内碱基频率,我们以参考基因频率差异绝对值不小于0.5为标准,以100 kb为窗口,10 kb为步长,计算组间窗口差异变异数目。为筛选候选基因,本研究以群体间遗传分化指数 F_{st} (Fixation index)、欧式距离ED² (Euclidean distance)和基因组间隔内差异SNP (Single Nucleotide Polymorphisms)数量三者前1%为筛选标准,将3种方案交集区域作为基因组潜在抗病区域,区域内的注释基因作为候选的潜在抗病相关基因[33]。

1.9 转录组联合筛选及验证

利用之前所获得的H和A⁺系在人工感染前后的转录组数据[34],将转录组人工感染前后的差异表达基因与潜在抗病区域内的注释基因进行联合分析,筛选位于潜在抗病区域内且在人工感染前后有差异表达的基因。随后对这些差异表达基因在FZ2、H和A⁺系人工感染前后48h的头肾样品中,进行qPCR的验证,qPCR的方法如上所述。

2 结果

2.1 雌核生殖克隆系构建和微卫星鉴定

通过搜集方正银鲫、鄱阳银鲫和彭泽鲫等地理种群的活体样本,在方正银鲫中随机挑选了3尾体型大的雌性个体,在鄱阳银鲫中挑选了1尾体型大的雌性个体,在彭泽鲫中也挑选了1尾体型大的雌性个体。利用兴国红鲤精子对这5尾个体进行了雌核生殖,构建了5个雌核生殖克隆系:方正银鲫克

隆系1 (FZ1)、方正银鲫克隆系2 (FZ2)、方正银鲫克隆系3 (FZ3)、鄱阳银鲫克隆系(PY)和彭泽鲫克隆系(PZ)。同时用高抗品系H系和易感品种异育银鲫“中科3号”(A⁺系) 2个克隆系作为对照(图 1A)。随后对这7个克隆系的母本进行了微卫星鉴定(图 1B), 总共使用了7对微卫星引物, 其中微卫星引物 YJ0004^[20]、HLJYJ029^[23]和 GCE401^[23]共同使用能够很好的区分这7个克隆系(图 1B)。

2.2 不同克隆系疱疹病毒抗性检测

7个克隆系在相同水体的不同网箱中养殖4个月, 对7个克隆系分别取30尾个体, 进行鲫疱疹病毒人工感染试验, 随后每日统计存活情况。人工感染试验共进行2次, 两次结果相近: 高抗对照克隆系H始终没有出现死亡现象, 而易感对照克隆系A⁺感染后最先出现死亡现象且存活率最低; 感染后的病鱼出现游泳能力下降及鳍条基部与腹部出血等症状; 直至感染病毒后14—21d, 各克隆系均不再出现死亡情况。这些结果说明人工感染试验结果稳定可靠。在构建的5个地理种群克隆系中, 克隆系FZ2表现出与高抗克隆系H类似的高存活率, 克隆系PY显示出中等存活率, 而克隆系FZ1、FZ3和PZ呈现出类似易感克隆系A⁺的低存活率。存活率由高到低依次为H (100%) > FZ2 (95%) > PY (40%) > FZ1 (20%) = FZ3 (20%) = PZ (20%) > A⁺ (10%) (图 2)。

对攻毒后48h的鱼体肝脏组织进行取样, 每个克隆系各取5尾, 制作的HE染色的病理切片结果显示: 在克隆系H和FZ2中, 肝脏细胞排列紧密, 细胞核形态大小正常。而在克隆系FZ1、FZ3、PZ和A⁺中, 细胞核表现为扩张性改变, 随后核膜完整性丧失, 伴随染色质和核内物质的释放, 细胞结构松散(图 3A)。同时对鲫疱疹病毒基因 $orf72$ 在肝脏中的表达量进行了分析, 通过其表达量可以反映病毒载量。本文发现在克隆系H和FZ2中, $orf72$ 表达量较低; 在克隆系FZ1、FZ3、PZ和A⁺中, $orf72$ 表达量较高(图 3B)。这些结果说明, 克隆系FZ2与高抗克隆系H类似, 鲫疱疹病毒抗性较强; 克隆系FZ1、FZ3和PZ与易感克隆系A⁺类似, 对鲫疱疹病毒易感。

2.3 基因组重测序

对以上7个克隆系各10尾(共70尾)个体进行了全基因组重测序, 获得原始数据(Raw data)的reads数为7857.20 M, 质控后有效数据(Clean data)的reads数为7553.11 M, 有效数据reads数占原始数据reads数的比例为93.95%—97.84% (平均比例96.13%)。质控后有效数据的数据量为1119.37 Gb。以雌性银鲫基因组为参考(GenBank: GCA_019843895.2)^[3], 有效数据的reads比对到参考基因组的比例为97.85%—99.55% (平均比对率99.36%), 每个样品的基因组平均覆盖深度处于8.66×—12.56× (平均覆盖

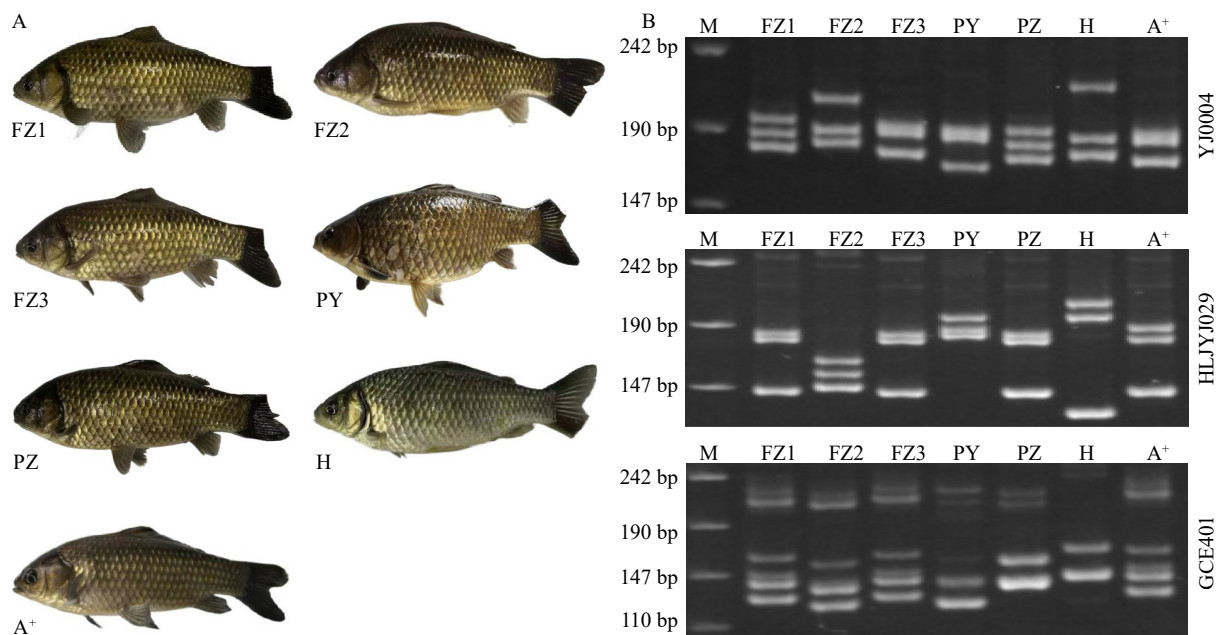


图 1 不同银鲫克隆系母本的形态观察及微卫星鉴定

Fig. 1 Morphological observation and microsatellite identification in different gynogenetic clones of gibel carp

A. 不同银鲫克隆系母本形态特征; B. 微卫星鉴定, 引物编号分别为YJ0004、HLJYJ029和GCE401; FZ1、FZ2、FZ3、PY、PZ、H和A⁺分别表示克隆系的编号; M. Marker

A. Morphological characteristics in different gynogenetic clones; B. Microsatellite identification; Primer numbers are YJ0004, HLJYJ029, and GCE401, respectively. FZ1, FZ2, FZ3, PY, PZ, H, and A⁺ represent the clone designations; M. Marker

度 $9.65\times$)。基于序列比对结果, 利用GATK^[35]进行SNP调用, 得到71216586个SNP, 进行质量过滤获得70444072个SNP。为了保证不同克隆系群体分析的稳定性, 利用VCFtools^[30]进行了群体过滤, 只保留了次等位频率大于0.05, 位点缺失率小于25%, 最小平均深度是5的二等位基因位点, 最终获得30936854个SNP用于后续全基因组关联分析。

2.4 潜在抗病相关区域筛选

将人工感染后存活率高的H和FZ2克隆系设定为高抗群体, 人工感染后存活率低的FZ1、FZ3、PZ和A⁺克隆系设定为易感群体。对这两个极端群体进行了分离性状定位分析PSA (Phenotype segregation analysis)。首先计算了群体间遗传分化指数 F_{st} (Fixation index), F_{st} 值越高, 表明该位点在不同

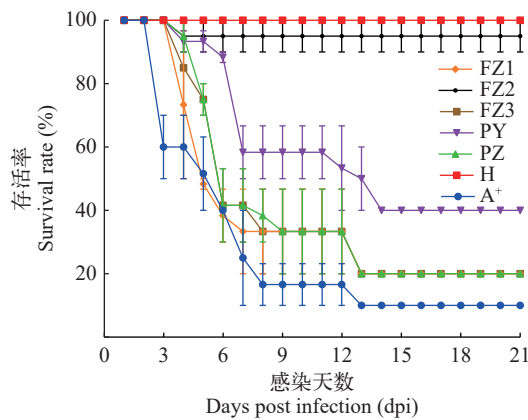


图2 不同银鲫克隆系感染鲫疱疹病毒后存活率

Fig. 2 Survival rate in different gynogenetic clones of gibel carp following infection with CaHV

横坐标表示感染后的天数, 纵坐标表示存活率

The x-axis represents the days post infection, and the y-axis represents the survival rate

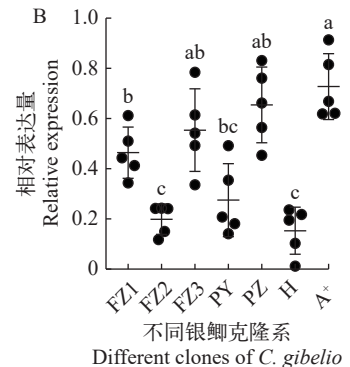
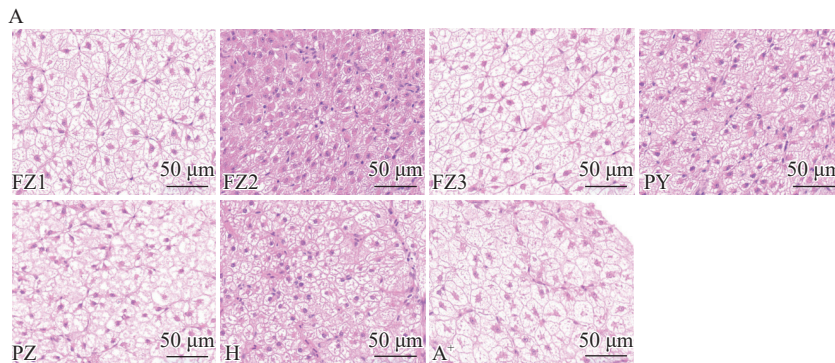


图3 感染鲫疱疹病毒后肝脏组织病理切片及肝脏中病毒基因 $orf72$ 的转录表达

Fig. 3 Liver histopathological sections and transcriptional expression of the viral gene $orf72$ following infection with CaHV

A. 感染鲫疱疹病毒48h后的肝脏病理组织切片; B. 病毒基因 $orf72$ 的转录活性检测; 不同的小写字母表示具有统计上的显著性差异 ($P < 0.05$)

A. Liver histopathological sections at 48h after infection with CaHV; B. Transcriptional expression of the viral gene $orf72$ in liver at 48h after infection with CaHV; Different letters represent statistical differences ($P < 0.05$)

极端群体之间的遗传分化程度也越大(图4A)。随后利用每个等位基因的深度, 计算欧氏距离ED (Euclidean distance), 群体间的欧氏距离越大, 差异也就越大(图4B)。此外, 如果变异与性状相关, 在极端性状的两个群体间, 变异的频率应该存在差异。因此, 基于差异变异数目分析, 还绘制了整个基因组间隔内差异SNP数量(Number)的分布情况(图4C)。以上的3个分析都是用VCFtools软件^[30], 以100 kb为窗口, 10 kb为步移长度进行的。本研究同时选取了以上3种方法筛选得到的前1%的基因组区间, 并做交集, 总共获得63个基因组区间, 这些基因组区域被认为是潜在的抗病相关区域。

2.5 潜在抗病相关基因筛选

在潜在的抗病相关基因组区域中, 总共包含了213个注释基因。对这些基因进行KEGG (Kyoto Encyclopedia of Genes and Genomes)通路富集分析, 发现排名前20的通路中存在很多与免疫反应相关通路, 比如NOD样受体信号通路、C型凝集素受体信号通路、趋化因子信号通路、补体和凝血级联、TRP通道的炎症介质调节、动物自噬、白细胞经内皮细胞迁移等通路(图5A)。随后利用前期发表的克隆系H和克隆系A⁺在人工感染前后的转录组数据^[34], 把人工感染前后的差异表达基因与213个注释基因进行联合分析, 绘制了韦恩图(图5B)。在潜在抗病相关区域的213个注释基因中, 有23个基因在克隆系H或者克隆系A⁺转录组数据中呈现出人工感染前和人工感染后的差异表达(图5C)。随后对这23个基因在高抗系H和FZ2, 以及易感系A⁺中进行了荧光定量PCR验证(图5D)。其中, *rasef-A* (Ras and EF-Hand domain containing protein)、*ccn1l2-B* (cellular communication network factor 1,

like 2)、*ptpn12-A* (tyrosine-protein phosphatase non-receptor type 12)、*bgn-A* (biglycan)、*anxa5-B* (annexin

A5)、*gpa33a-B* (glycoprotein A33 a)、*dnase2-B* (deoxyribonuclease-2)、*antxr1b-B* (anthrax toxin receptor

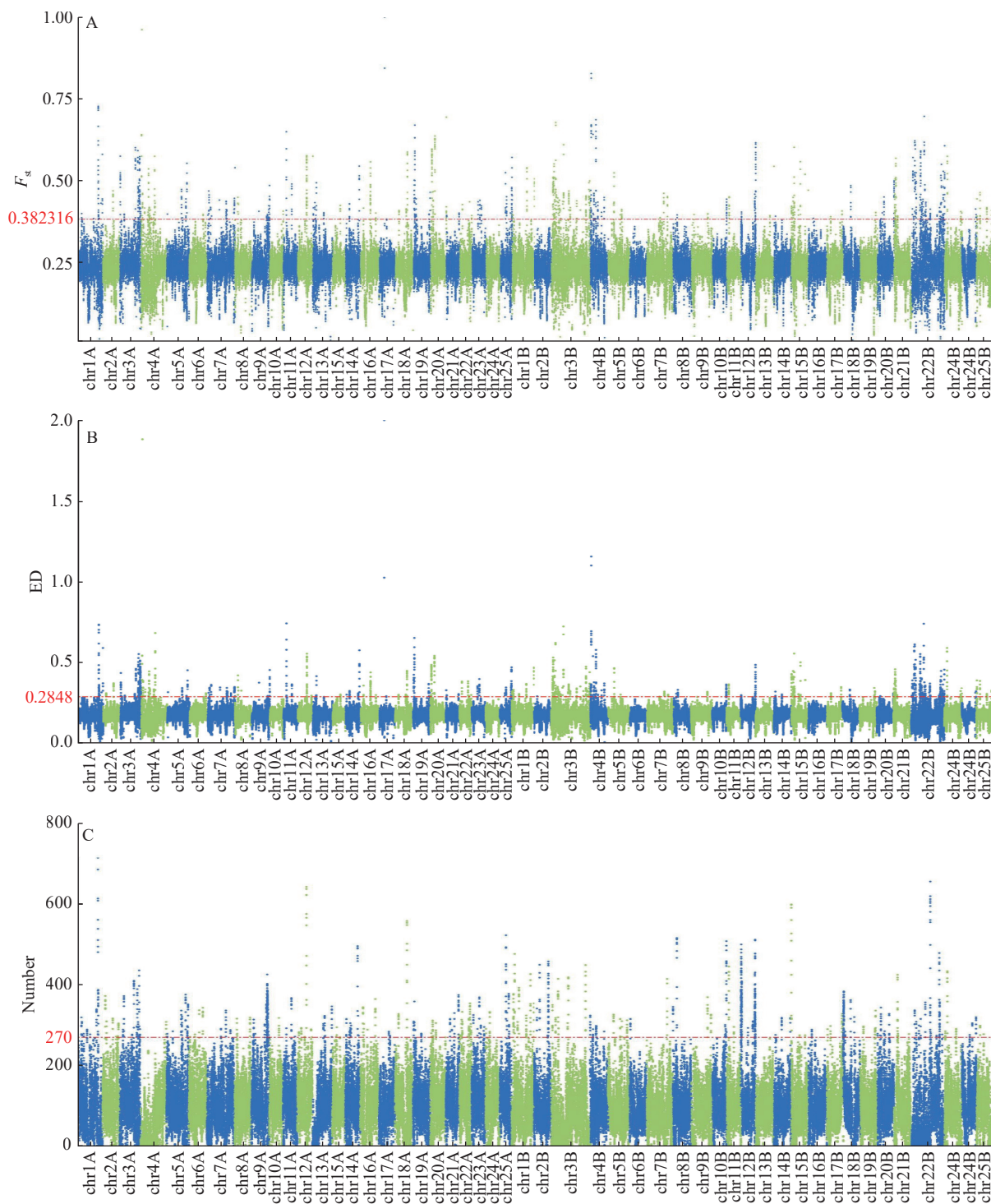


图4 全基因组关联分析抗病性状相关位点的曼哈顿图

Fig. 4 The Manhattan plot of antiviral resistance-related loci in whole genome association analysis

A. 高抗和易感群体间遗传分化指数(F_{st})分析, 虚线是相应阈值为0.382316的位置; B. 欧氏距离(ED)分析, 虚线是相应阈值为0.2848的位置; C. 基因组窗口内差异SNP数量(Number)分析, 虚线是相应阈值为270的位置; 基因组SNP分析以100 kb为窗口

A. Fixation index (F_{st}) between highly resistant and susceptible populations; The dashed line represents the position with a corresponding threshold of 0.382316; B. Euclidean distance (ED) between highly resistant and susceptible populations; The dashed line represents the position with a corresponding threshold of 0.2848; C. The number of differential SNPs within genomic windows; The dashed line represents the position with a corresponding threshold of 270; SNP analysis with a window size of 100 kb

1b)、*ctsh-B* (cathepsin H)、*phyhd1-B* (phytanoyl-CoA dioxygenase domain-containing protein 1)和*kcnh1a-B* (potassium voltage-gated channel subfamily H member 1a)等11个基因的荧光定量结果与转

录组数据的变化趋势一致(图 5C和5D); 而剩余12个基因的荧光定量PCR结果与转录组数据不一致。在这11个基因中,*rasef-A*和*ccn1l2-B*在病毒感染后均上调表达,*ptpn12-A*、*bgn-A*、*anxa5-B*、*gpa33a-B*

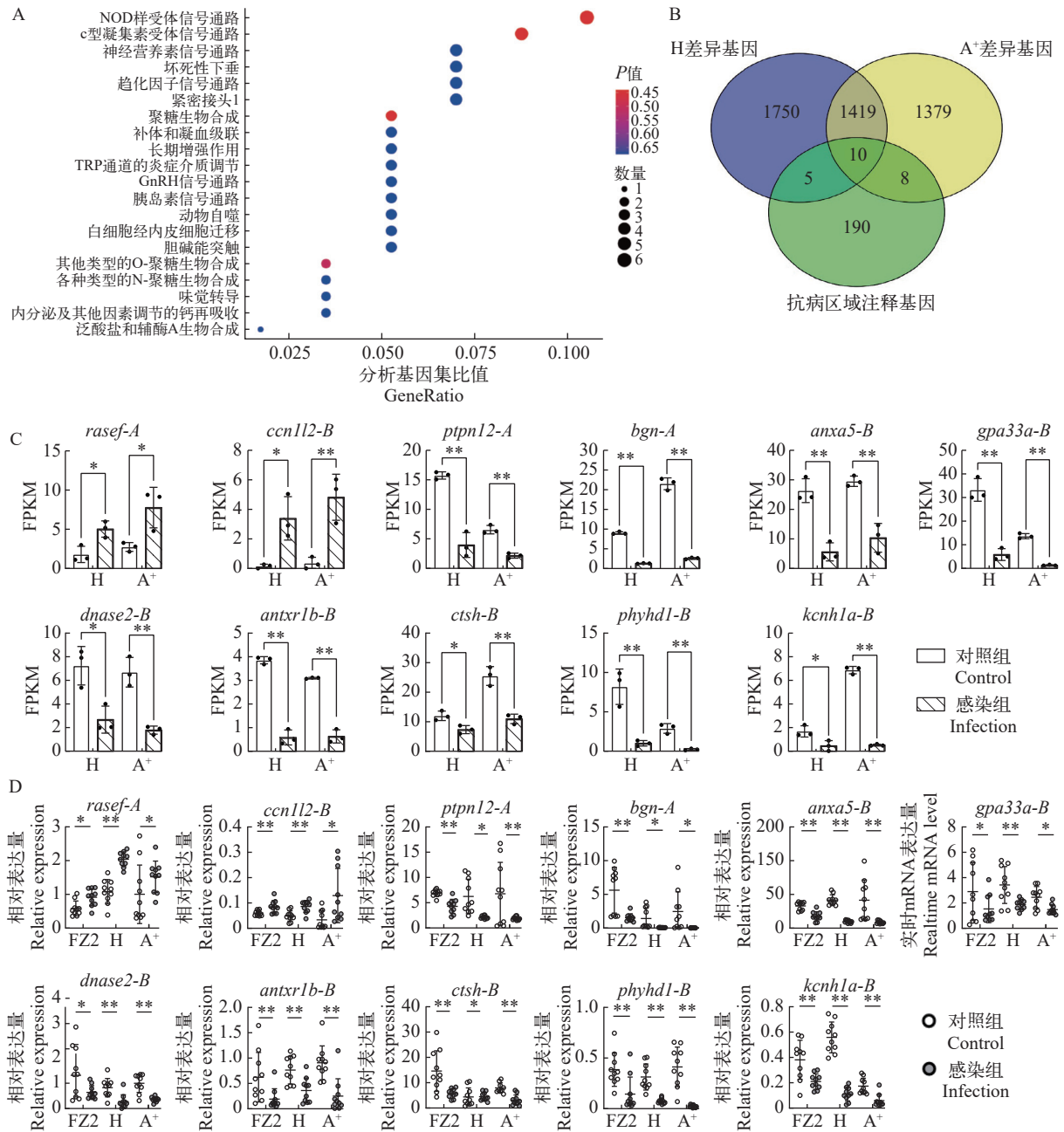


图 5 潜在抗病相关基因的鉴定

Fig. 5 Identification of potential antiviral resistance-related genes

A. 抗病相关基因组区域213个注释基因的KEGG功能富集分析; B. 克隆系H和A⁺病毒感染前后差异基因与抗病区域注释基因的韦恩图; C. 11个潜在抗病相关基因在克隆系H和A⁺病毒感染前后转录组数据中的表达变化; D. 11个潜在抗病相关基因在FZ2、H和A⁺系中病毒感染前后的qPCR验证; $P>0.05$ 表示差异不显著, $*P<0.05$ 表示差异显著, $**P<0.01$ 表示差异极显著

A. KEGG functional enrichment analysis of the 213 annotated genes in the antiviral resistance-related genomic regions; B. Venn diagram of differentially expressed genes (DEGs) before and after infection in clone H, DEGs before and after infection in clone A⁺; C. Expression changes of 11 potential disease resistance-related genes in transcriptomic data before and after infection in H and A⁺ clones candidate genes in the transcriptome; D. qPCR validation of 11 potential antiviral resistance-related genes before and after infection in FZ2, H and A⁺ clones. $P>0.05$ indicates no significant difference, $*P<0.05$ indicates significant difference, $**P<0.01$ indicates extremely significant difference

B、*dnase2-B*、*antxr1b-B*、*ctsh-B*、*phyhd1-B*和*kcnh1a-B*在病毒感染后均下调表达。这些结果暗示着以上11个基因可能参与鲫疱疹病毒感染过程,是潜在鲫疱疹病毒抗性相关基因。

3 讨论

银鲫在我国分布广泛,不同地理种群遗传差异较大,为筛选具有抗病性状的优良种质提供了丰富的资源^[1]。本研究搜集了方正银鲫、鄱阳银鲫和彭泽鲫等地理种群,并在其中挑选具有体型优势的个体构建了5个雌核生殖克隆系,同时以已知抗性的H和A⁺系为对照,通过人工感染试验筛选出具有强鲫疱疹病毒抗性的方正银鲫克隆系FZ2。通过全基因组关联分析,筛选到63个抗病相关区域,其中包含有213个注释基因。此外,通过与转录组联合分析和荧光定量PCR验证,鉴定到11个潜在抗病相关基因,为后续银鲫抗病遗传育种工作奠定了基础。

有性生殖鱼类会发生减数分裂及随后的核子融合,其繁育家系子代个体间存在遗传分化,可以在家系内部筛选具有不同遗传信息及不同性状的个体,用于后续的遗传选育。比如,基于两性物种的混合家系,利用全基因组选育在大黄鱼上已经获得很好的效果^[36]。然而,银鲫进行天然雌核生殖,在卵子成熟过程中,不发生正常的减数分裂,不减数的配子在精子的刺激下,形成与母本遗传信息一致的子代^[3]。雌核生殖家系的子代间几乎没有遗传差异,相当于是母本的克隆^[22]。因此,雌核生殖银鲫不能像两性物种一样在家系内部进行选育。通常可以将一个雌核生殖克隆系视为一个整体,在不同克隆系间进行比较和筛选,从而找出候选克隆系进行下一步的遗传选育。本研究发现克隆系FZ2与克隆系H类似,具有较强的鲫疱疹病毒抗性。但是H系的体型小、生长慢、饲料转换率低,不适合作为养殖品种培育。而FZ2是来自方正县的地理种群中筛选的体型较大的亲本所繁育的克隆系,且在网箱养殖过程中也展示出了较好的生长性状,具有一定的开发前景。后续进一步的抗病性能测试及池塘养殖条件下的生长性能测试在FZ2中也亟待开展。

3.1 抗病相关基因*rasef-A*和*ccn1l2-B*的功能与疾病研究

在筛选到的11个潜在抗病相关基因中,*rasef-A*和*ccn1l2-B*在病毒感染后上调表达。*rasef*编码GTP酶,可能参与囊泡运输或其他细胞过程。在乳腺癌^[37]、直肠癌^[38]、黑色素瘤^[39]等癌症组织中*Rasef*的修饰或者表达都出现了异常。*rasef*被认为是一

个抑癌基因,可能通过参与DNA修复过程抑制结直肠癌的发生发展^[38]。*Ccn1l2*是一种分泌型基质细胞蛋白^[40-42],具有促炎作用。类风湿性关节炎滑液中含有高水平的*Ccn1l2*,可有效刺激成纤维细胞样滑膜细胞增殖,并且这种作用被人*Ccn1l2*的中和抗体消除。类风湿性关节炎成纤维细胞样滑膜细胞中*ccn1l2*的表达能被白细胞介素17通过核因子κB (Nuclear factor kappa-B, NF-κB)和丝裂原活化蛋白激酶(p38 mitogen-activated protein kinase, p38 MAPK)信号通路上调,敲低*ccn1l2*的表达可显著抑制白细胞介素17刺激的成纤维细胞样滑膜细胞增殖^[43]。另外,*ccn1l2*在银屑病皮损表皮和关节处的表达强度与疾病严重程度显著相关。在增生的表皮中过表达的*ccn1l2*通过刺激趋化因子配体20的产生来增加银屑病影响的皮肤病变的炎性细胞浸润^[44]。此外,弗里德伯格等^[45]和Lowes等^[46]认为*ccn1l2*改变的IL-1是银屑病炎症过程和角质形成细胞活化的始动者。这些发现都说明了*ccn1l2*在银屑病中的促炎功能。此外,*ccn1l2*还被发现介导神经炎症参与新生儿缺氧缺血性脑损伤^[47]及参与原发性胆汁性胆管炎(PBC)的发病过程^[48]。在银鲫*ccn1l2*是否也通过促炎的作用参与到抵抗鲫疱疹病毒感染还有待进一步研究。

3.2 病毒感染后下调的9个潜在抗病基因及其功能与免疫调控分析

在筛选到的11个潜在抗病相关基因中,除了*rasef-A*和*ccn1l2-B*,其他9个基因在病毒感染后均下调表达。其中,*Ptpn12*在小鼠中能够调控树突状细胞的迁移、T细胞依赖性免疫和自身免疫,是诱导抗原特异性T细胞反应能力的关键调控因子^[49]。*Bgn*是一种细胞外基质蛋白,在介导上皮细胞的形态、生长、分化和迁移中起着至关重要的作用^[50]。*Bgn*在具有免疫抑制特性的肿瘤区域特异性高表达,并与结肠癌中多种免疫细胞的浸润密切相关,尤其是M2型巨噬细胞和调节性T细胞^[51]。*Anxa5*是一种在正常胎盘中大量表达的蛋白,被认为具有抗凝血功能^[52]。*Gpa33a*最初在非洲爪蟾胸腺细胞表面被鉴定到,属于免疫球蛋白超家族成员,在B细胞、树突状细胞、自然杀伤细胞和固有淋巴样细胞中表达,并且在CD4⁺T细胞亚群中表达最为显著^[53]。*Dnase2*是一种溶酶体酶,负责在红细胞生成或凋亡过程中降解细胞释放的DNA。*Dnase2*基因敲除小鼠表现出与人类类风湿性关节炎相似的慢性多发性关节炎^[54]。*Antxr1b*是一种具有胞外结构域的跨膜蛋白,与骨形成过程密切相关,在血管生成中发挥重要作用。*antxr1b*基因在破骨细胞分化及破骨

细胞介导的内皮细胞血管生成中的作用非常重要^[55]。*ctsh*基因敲除会扰乱有氧糖酵解过程并增强有氧呼吸, 通过上调和释放促凋亡因子促进细胞凋亡^[56]。*Phyhd1*与阿尔茨海默病、一些癌症和免疫细胞功能有关, 在细胞核和细胞质中均有表达^[57]。利用荧光定量PCR分析小鼠*Phyhd1*时发现该基因在T细胞和B细胞中大量表达, 并且与T细胞分化和效应T细胞的功能有关^[58]。*Kcnh1a*主要分布于人体的大脑、成肌细胞和肾上腺及生殖相关器官^[59]。有研究表明*kcnh1a*可能在妊娠期宫颈血管稳态的调节中发挥作用^[60]。在上述的这些基因中,*ptpn12*、*bgn*、*gpa33a*和*phyhd1*与T细胞发挥功能相关;*anxa5*、*antxr1b*和*kcnh1a*与血管稳态相关, 暗示着这些基因可能与银鲫抗病毒免疫反应及感染后的出血症状有关。

3.3 荧光定量PCR与转录组数据不一致的12个基因及其在免疫调控和疾病中的潜在作用

除了已经筛选到的11个基因外, 还有12个基因的荧光定量PCR结果与转录组数据不一致。其中, C3主要由肝细胞和巨噬细胞产生, 是血清中的核心成分, 它可以调节T细胞功能, 在银屑病中发挥重要作用^[61]。*Nectin3*是一种细胞黏附分子, 与乳腺癌、肺癌和胰腺癌等疾病的不良预后因素有关^[62, 63], 并且在卵巢癌中具有促进肿瘤进展的作用^[64]。*Svild*是凝溶胶蛋白家族成员之一, 参与细胞增殖、运动和信号转导等过程^[65, 66], 同时也参与脂多糖诱导的巨噬细胞炎症反应^[67]。*Cfh1l*与人类中性粒细胞^[68]和人类免疫系统^[69]有关。*Crata*是一种关键的线粒体酶, 能够参与并维持代谢稳态^[70]。*Crata*影响细胞膜的脂质组成, 并调节心肌^[71]中免疫细胞的活化, 改变*Crata*的表达可以改变免疫细胞内脂质中间产物的平衡, 加剧炎症反应, 促进肥胖和糖尿病等代谢性疾病^[71, 72]。*Agtrap*在结肠癌中与不良预后呈正相关^[73], 同时在胃癌中检测到*Agtrap-braf*基因融合, 说明*Agtrap*可能在肿瘤进展中发挥作用^[74]。也有研究表明, *Agtrap*与免疫浸润和T细胞耗竭呈正相关^[75]。*Comtd1*蛋白定位于色素细胞中的线粒体, 在小鼠黑色素细胞系中敲除*Comtd1*会导致褐黑色素代谢物减少, 并导致谷氨酸/谷胱甘肽、核黄素和三羧酸循环代谢物显著改变^[76]。*Pacs2*在胰岛素抵抗中调节细胞能量代谢^[77], 可以保护小鼠肾脏血管功能。此外, *Pacs2*在肾小球内皮细胞和糖尿病肾病中调节游离脂肪酸代谢^[78]。*Ahsg1*糖蛋白是一种多功能蛋白^[79], 在乳腺癌、结直肠癌、肺癌、肝癌、头颈部肿瘤中存在差异表达^[80]。血清中*Ahsg1*通过阻断转化生长因子 β (TGF- β)与细胞表面受体结合, 抑制TGF-

β 信号转导, 抑制TGF- β 诱导的上皮-间质转化, 从而抑制肿瘤进展^[81-86]。虽然这12个基因的荧光定量PCR结果与转录组数据不一致, 但这些基因在多种生理和病理过程中发挥着关键作用, 涉及免疫调控、代谢稳态、肿瘤发生及炎症反应等多个方面, 同样值得进一步深入研究。

综上所述, 本研究构建了7个银鲫雌核生殖克隆系, 并对其进行了疱疹病毒抗性差异研究, 鉴定出1个具有较强疱疹病毒抗性的方正银鲫克隆系FZ2; 同时通过全基因组关联解析筛选到63个与抗病性状相关的基因组区域。联合转录组分析和荧光定量PCR验证, 鉴定到11个潜在抗病相关基因。本研究不仅为银鲫抗病育种工作提供了优良种质, 还为疱疹病毒的精准防控靶点提供了参考。

(作者声明本文符合出版伦理要求)

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RESISTANCE DIFFERENCES TO *CARASSIUS AURSTUS* HERPESVIRUS AND THE GENOME-WIDE ASSOCIATION STUDY AMONG DIFFERENT CLONES OF GIBEL CARP

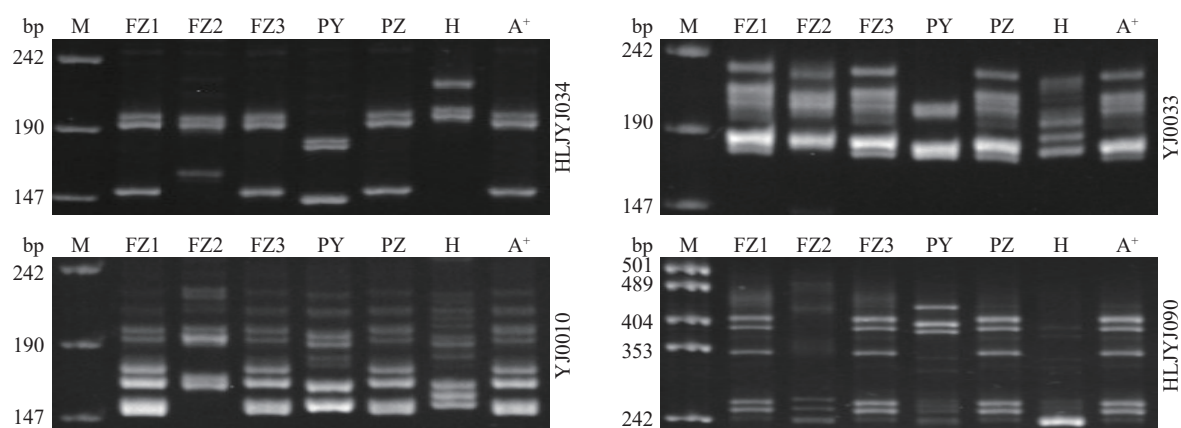
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Abstract: To investigate the resistance differences against *Carassius auratus* herpesvirus (CaHV) among different gynogenetic clones of gibel carp (*Carassius gibelio*) and identify potential antiviral resistance-related genes, seven gynogenetic clones and performed microsatellite identification on their maternal individuals. These seven clones were established, including three Fangzheng clones (FZ1, FZ2, and FZ3), one Poyang clone (PY), one Pengze clone (PZ), along with two control clones: the highly resistant clone H and the susceptible variety allogynogenetic gibel carp “CAS III” (clone A⁺). Subsequently, artificial infection experiments with CaHV were conducted on these clones. The survival rates, ranked from highest to lowest, were: H (100%)>FZ2 (95%)>PY (40%)>FZ1 (20%)=FZ3 (20%)=PZ (20%)>A⁺ (10%). At 48h post-infection, histological observations revealed that the liver cells of the highly resistant clones FZ2 and H exhibited tighter cellular arrangements and significantly lower expression levels of CaHV gene *orf72*, indicating low viral load. In contrast, susceptible clones FZ1, FZ3, PZ, and A⁺ displayed swollen liver cells and elevated *orf72* expression, suggesting high viral load. Additionally, whole-genome resequencing of 10 offspring from each clone identified 35278852 single nucleotide polymorphism loci. Genome-wide association study mapped 63 genomic regions associated with disease resistance traits, encompassing 213 annotated genes. Integrative analysis with transcriptomic data and qPCR validation identified 11 potential antiviral resistance-related genes. This study not only identifies superior germplasm with high resistance to CaHV but also screens potential genes for disease resistance, laying a foundation for antiviral disease-resistant breeding in gibel carp.

Key words: Gynogenesis; Clone; CaHV; Disease resistant breeding; Gibel carp

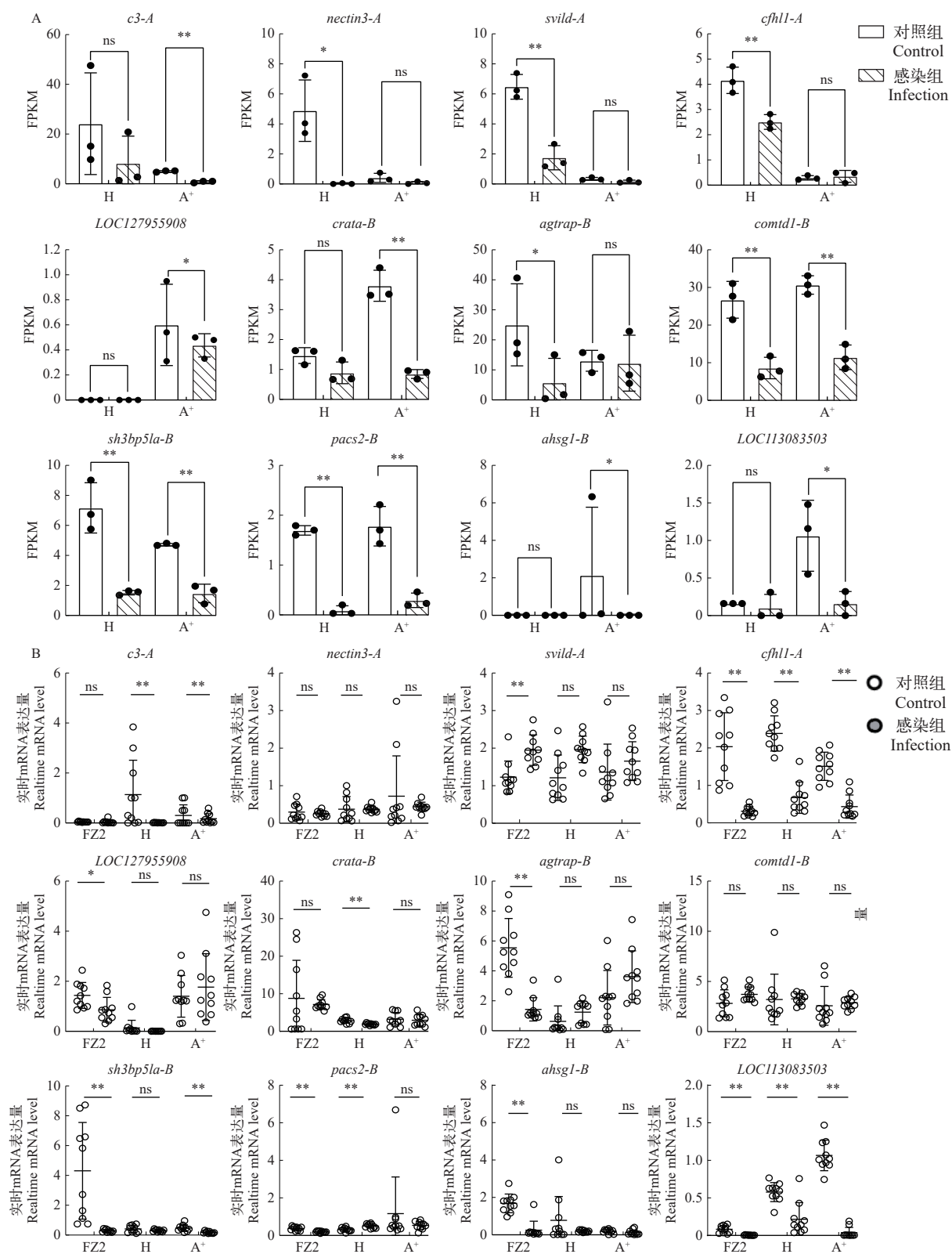


附图 S1 不同银鲫克隆系母本的4对微卫星标记鉴定结果

Appendix Fig. S1 Microsatellite identification in different gynogenetic clones of gibel carp using the other four primer pairs

引物编号分别为HLJYJ034、YJ0033、YJ0010和HLJYJ090

Primer numbers are HLJYJ034, YJ0033, YJ0010, and HLJYJ090



附图 S2 12个基因在转录组中的变化及qPCR验证

Appendix Fig. S2 Transcriptomic changes and qPCR validation of the remaining 12 genes

A. 12个基因在H和A⁺系中病毒感染前后转录组数据中的表达变化; B. 12个基因在FZ2、H和A⁺系中病毒感染前后的qPCR验证; ns. 没有显著差异, $P>0.05$ 表示差异不显著, $*P<0.05$ 表示差异显著, $**P<0.01$ 表示差异极显著

A. Expression changes of the remaining 12 genes in transcriptomic data before and after infection in H and A⁺ clones; B. qPCR validation of the remaining 12 genes before and after infection in FZ2, H and A⁺ clones; ns. no significance. $P>0.05$ indicates no significant difference, $*P<0.05$ indicates significant difference, $**P<0.01$ indicates extremely significant difference