クルマエビ Marsupenaeus japonicus 血球細胞の分子生物学的分類を目的とした基礎研究

博士学位論文

クルマエビ *Marsupenaeus japonicus* 血球細胞の分子生物学的分類を目的とした基礎研究

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目次

第 1 章　研究の背景

第 1 節 国内外における甲殻類，主にクルマエビ類養殖産業の位置づけ

現在地球上には約 75 億人もの人が暮らしているが，その人口は増加の一途をたどり，2050 年には 91 億人に達すると予測されている．世界の穀物生産量は毎年 26 億トン以上あり，在庫も考慮すると世界中の人が十分に食べられるだけの食料は生産されている (United States Department of Agriculture, 2018)．それにもかかわらず，2017 年でも 9 人に 1 人の割合である 8 億 2100 万人が飢餓に苦しんでいる (Food and Agriculture Organization of the UN, 2018a)．予測通りに世界の人口増加が進むと，食料問題は今よりもなお一層深刻になることは疑いようもない．穀物の十分な生産に反して食糧問題が発生する大きな原因として，穀物の畜産・養殖飼料への供給および製造，流通，調理や食事の際の廃棄が挙げられる (Food and Agriculture Organization of the UN, 2011)．約 3 割の穀物が廃棄されているため，それを減少させる努力は当然必要だが，同じく約 3 割の穀物が家畜や養殖魚の飼料として供給されている (Food and Agriculture Organization of the UN, 2018b)．供給される穀物を無駄なく動物性タンパク質の生産に活用するために，畜産・養殖技術を改善することは人類の課題として極めて重要なことである．

魚介類の養殖は鶏，豚および牛の畜産と異なり，成育時に必要な飼料がはるかに少なく効率的な動物性タンパク質の供給が見込まれるため拡大が期待されている (The World Bank, 2013)．魚介類の養殖生産量はここ 20 年で発展を続けており，2016 年には漁業も含めた全水産生物生産量のおよそ 1.4 億トン中の約 40%を占める 0.6 億トンを生産するに至っている (図 1)．さらに食用向け魚介類では 2014 年から養殖による生産量が天然の漁獲量を上回っている (国際農林業協働協会, 2017)．世界

の漁船漁業生産量は，1980 年代後半以降頭打ちとなっており，その背景には，多くの海洋水産資源が適正レベルの上限まで, 又はそれを超えて利用されるようになっていること，また，新たな資源の開発が困難となっていることが挙げられる (水産庁, 2016). 世界の漁獲量において 10 位までを占める資源の多くが既に満限まで利用されているか過剰漁獲となっており, 今後これらの魚種の漁獲量を持続的に大きく増やす余地はなく，漁業による生産量の拡大は見込まれない (水産庁, 2016). 魚介類による動物性タンパク質の供給は養殖生産のさらなる拡大にかかっていることは明白であり, 既存の施設や設備を最大限に活かすための効率の良い養殖手法の開発や病気による大量斃死の防除など基本的な課題の解決が必須である.

全水産生物生産量およそ 1.4 億トン中の約 8%，1100 万トンを占めるのは甲殻類の生産量である (図 2A). エビやカニなどを含む甲殻類は消費者の嗜好性が高いため市場での価値が高く，全水産生物により生み出される経済的価値の約 22%を占める (図 2B). さらに成長も早いため養殖対象種として非常に人気が高い. 中でもクルマエビ *Marsupenaeus japonicus*，ブラックタイガー*Penaeus monodon* およびバナメイエビ *Litopenaeus vannamei* を含むクルマエビ科のエビ (クルマエビ類) には大型のエビが多く世界各国で養殖が盛んであり, その養殖生産量は中国と東南アジアを中心に拡大し続けている (Food and Agriculture Organization of the UN, 2018a). 中国および東南アジアにおけるクルマエビ類の養殖はブラックタイガーが主な対象種であったが，その生産量は減少しており，バナメイエビに養殖対象種が移り変った. これは底生性のブラックタイガーに比べバナメイエビは遊泳性であるため同一スペースでより集約的な養殖ができること, ブラックタイガーに比べ病気に強いといわれていたこと, 昼間隠れるための砂を敷く必要がなく底層の管理が容易なこと

などが利点としてあげられるからである．高い利益を短期間で得られるため，集約的な養殖手法が中国や東南アジアを中心に急速に発展してきたバナメイエビ養殖だが，後述の病原細菌による疾病により 2013 年前後には一時その養殖生産量は減少した．しかしながら，近年はプロバイオティクスや汚泥の除去などにも工夫がなされるようになったほか，閉鎖循環式陸上養殖技術の導入も始まり，バナメイエビの養殖生産量は今後も拡大が見込まれる (養殖ビジネス編集部, 2015).

日本国内では，現在 2 種類のクルマエビ類が養殖されている．九州沖縄地方を中心として行われるクルマエビ養殖と新規参入事業としてのバナメイエビ養殖である．年間約 1600 トンが生産されている養殖クルマエビは，おがくずや保水性シートを用いることで鮮度を保ち生きた状態で輸送可能なことから，贈答品や高級料理店への供給が多い．さらに，冬を中心に漁獲される天然のものとは異なり，季節を問わず供給できることも魅力であり養殖でありながら高い価格 (平均単価 6,180-9,431 円/ kg, 2018 年 1 月から 2018 年 9 月の間) (東京都中央卸売市場市場統計情報, http://www.shijou-tokei.metro.tokyo.jp, accessed 2018-10-30) を保っている．一方のバナメイエビ養殖は，新潟県妙高市で IMT エンジニアリング株式会社が国内では初となる屋内型エビ生産システムを用いた養殖を 2007 年から開始している (マーシー・ワイルダー, 2014)．また，日本水産株式会社も 2018 年から九州地方において地熱を利用した閉鎖循環式陸上養殖システムを導入し，年間 100 トンの出荷を目指す本格的な事業として参入を果たすなど，2019 年現在も国内バナメイエビ養殖事業は拡大が続いている (日本水産株式プレスリリース, http://www.nissui.co.jp/news/20180719_2.html, accessed 2018-10-30)．これらのバナメイエビは妙高ゆきエビや白姫エビと命名され，ご当地ブランド化することで既存の

中国や東南アジア産冷凍バナメイエビとの差別化を図かり，人件費および光熱費の回収が図られている．この背景には前述の 2013 年前後の世界的なバナメイエビ生産量の減少によるエビ類の価格高騰および 2016 年に改定された水産資源保護法および持続的養殖生産確保法による国外からの活状態のクルマエビ類の輸入制限があり，国内での自給自足の機運が高まっていることが挙げられる．

近代的なエビ養殖は 1934 年に藤永元作がクルマエビの人工ふ化を成功したことに始まり，幼生の飼育方法確立後，クルマエビ養殖が国内で初めて商業的に行われるようになった．その後，藤永の下で学んだ台湾留学生の廖一永が 1968 年にブラックタイガーの人工飼育に成功し，台湾のエビ養殖の基礎を築いた．その後，台湾ではブラックタイガーの養殖が盛んに行われるようになったが，1980 年代の後半に養殖場の環境問題や疾病のまん延によりその生産量は著しく減少した．しかし，養殖技術は他の東南アジアの国々へと広がっていき現在のエビ養殖産業の発展に繋がっている (水産庁, 2014).

食の安全が求められる昨今，種苗生産から一貫して人の管理下で成長した養殖魚は安全性が高いと考えられ，認証制度の制定も進んでいる (Aquaculture Stewardship Council 2018, https://www.asc-aqua.org, accessed 2018-10-30; Seedlings Council for Sustainable Aquaculture, https://www.scsa.or.jp, accessed 2018-10-30)．これら背景からも今後ますますクルマエビ類のみならず養殖魚の生産量は増加していくと考えられる．

第２節 クルマエビ類に関わる疾病について

エビ養殖の歴史は病原微生物との戦いの歴史といっても過言ではない．甲殻類の

養殖生産量と“shrimp & disease”のキーワードで検索した際の論文出版数を比較すると, 養殖生産量が増加するにつれて関連論文数が増加していることがみてとれる (図 3). エビ類は世界中で養殖が盛んであり，国境を超えて生きたエビの輸出入が行われるため，病原微生物の往来は避けなければいけない (Karunasagar and Ababouch 2012; Thitamadee *et al.*, 2016). そこで国際獣疫事務局 (World Organisation for Animal Health; OIE) の水生動物衛生規約委員会 (Aquatic Animal Health Standards Commission) では，輸出入の際に確認義務のある特定疾病 (OIE-Listed disease) として 9 種類の甲殻類の疾病，acute hepatopancreatic necrosis disease (急性肝膵臓壊死症)，infection with *Aphanomyces astaci* (アファノマイセス症)，infection with *Hepatobacter penaei* (壊死性肝膵炎)，infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV) (伝染性皮下・造血器壊死症)，infection with infectious myonecrosis virus (IMNV) (伝染性筋壊死症)，infection with Macrobrachium rosenbergii nodavirus (ホワイトテール病)，infection with Taura syndrome virus (TSV) (タウラ症候群)，infection with white spot syndrome virus (WSSV) (ホワイトスポット病) および infection with yellow head virus genotype 1 (YHV) (イエローヘッド病) を挙げている (OIE-Listed diseases, infections and infestations in force in 2018, http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2018/, accessed 2018-10-30). これらの中でも急性肝膵臓壊死症，壊死性肝膵炎，伝染性皮下・造血器壊死症，伝染性筋壊死症，タウラ症候群，ホワイトスポット病およびイエローヘッド病の 7 疾病はクルマエビ類への感染が確認されており, これまでにも甚大な被害をもたらしてきた.

急性肝膵臓壊死症は 2009 年に中国で初めて感染が確認された細菌性の疾病であ

る (Tran *et al.*, 2013)．本疾病はその後，東南アジア諸国や中南米へも侵入し，多くの養殖業者に大きな被害を与えた結果，2013 年前後に世界的なエビ類の価格高騰を引き起こした．急性肝膵臓壊死症の主な症状として，肝膵臓の萎縮，蒼白，壊死または成育不良が報告されている．本疾病は，池入れ後の稚エビに対して 100%の致死率を 30 日以内に示すことから，早期死亡症候群 (Early Mortality Syndrome: EMS) と発見当初は呼ばれていた (De Schryver *et al.*, 2014; Lightner *et al.*, 2012)．Trang ら (2013) が本疾病の原因細菌がビブリオ属細菌 *Vibrio parahaemolyticus* であると特定してから，多くの研究グループによってゲノム配列の決定が行われた (Gomez-Gil *et al.,* 2014; Han *et al.,* 2015a; Kondo *et al.,* 2014; Yang *et al.,* 2014)．その結果，急性肝膵臓壊死症を引き起こす *V. parahaemolyticus* が毒素遺伝子をコードする特有のプラスミドを保有することが明らかとなった．原因細菌および原因毒素遺伝子の特定以降は，PCR による毒素遺伝子検出手法 (Han *et al.*, 2015b; Sirikharin *et al.*, 2015; Tinwongger *et al.*, 2014) が開発された他，プロバイオティクス (Chomwong *et al.,* 2018; Chumpol *et al.,* 2017) や汚泥の清掃による本細菌の増殖抑制，養殖開始前の池を淡水で洗浄し空干しすることなどによる防除が進んでいる．さらに，鶏卵卵黄抗体 (Immunoglobulin Yolk: IgY) を用いた原因毒素の中和手法や (中村, 2018) ファージを用いた原因細菌の中和手法 (Jun *et al.*, 2018; Lomelí-Ortega and Martínez-Díaz 2014) の開発も行われている．

他の 6 種類の特定疾病はいずれもウイルスが原因である感染症であるため，抗生物質などの化学療法により感染エビを治療することや，水域に侵入したウイルスを完全に排除することは困難である．ウイルスは一度感染すると宿主内で急速にウイルス自身の数を増やし，新たな宿主へと感染を拡げる．そのためクルマエビ類の集

約的養殖下では爆発的な斃死が起こり得る．ホワイトスポット病は 1992 年に台湾北部で初めて疾病の発生が確認された二本鎖 DNA ウイルス WSSV を原因とするウイルス病であり (伊丹 2012; Chou *et al.,* 1995; Escobedo-Bonilla *et al*., 2008; Inada *et al.*, 2017)，現在までに日本，アメリカやオーストラリアを含むほぼ世界中のクルマエビ類養殖場での発症が確認されているクルマエビ類養殖に最も被害を与えている病原ウイルスである．WSSV の病原性は高く死亡率は 80-90%に達する．主な症状として，摂餌量の減退および行動緩慢が見られ，外骨格のクチクラ層に白点が観察される (伊丹 2012)．日本への侵入は中国からの種苗の輸入が原因であると考えられており，1993 年の発見当初は penaeid acute viremia (PAV) として報告されていた (Nakano *et al.*, 1994; Inada *et al.,* 2017; Takahashi *et al.*, 1994)．1990 年代のクルマエビ養殖はバブル崩壊のあおりを受け多くの養殖業者の経営がすでに傾いていたが, ホワイトスポット病による大量斃死は大きな打撃となり多数のクルマエビ養殖業者を廃業させる要因にもなった. ホワイトスポット病に対してはリコンビナントおよび DNA ワクチン (Kono *et al.,* 2009; Kono *et al.,* 2014; Namikoshi *et al.,* 2004; Witteveldt *et al.,* 2004)，プロバイオティクス (Leyva-Madrigal *et al.,* 2011; Peraza-Gómez *et al.,* 2009) や耐病性系統の作出 (Cock *et al.*, 2009) など様々な防除手法が開発されているが，いずれも実用可能なレベルには達していない．

その他 5 種類のウイルスは, 現在までに日本国内での養殖クルマエビにおける発症の報告はないが, 食用冷凍エビとして東南アジアから日本やアメリカに輸出されたエビから IHHNV および YHV が PCR 法により検出された事例がある (伊丹 2012)．水産資源保護法および持続的養殖生産確保法の下，海外からの輸入エビを監視し今後もこれらウイルスを国内に侵入させないようにすることは非常に重要

である．

さらに，*Enterocytozoon hepatopenaei* 感染症 (Tang *et al.,* 2015) や潜伏死病 (covert mortality nodavirus 感染症) (Zhang *et al.,* 2014) などといった新たな感染症の報告も相次いでおり，クルマエビ類の養殖に経済的な被害をもたらす病原細菌の報告は養殖生産量の拡大とともに増加することが予測される．

疾病は病原微生物，環境および宿主の 3 つの要因のバランスが崩れた際に発生する．そのため疾病の発生を抑制するには，それぞれの要因を適切に管理すること，すなわち，病原微生物の早期発見や養殖場への侵入経路の遮断，水質の管理や汚泥の清掃，また，宿主免疫の賦活化やワクチンによる予防を行うことが重要である．病原微生物の検出手法や養殖池の水質管理方法などは，他の生物に対してこれまで得られた手法を応用することで比較的開発が進んでいる．しかしながら，クルマエビ類の疾病に対するワクチンの開発や耐病性系統の作出などは研究レベルで一定の効果を発揮しているが (Cock *et al.*, 2009; Johnson *et al.*, 2008; Kono *et al.,* 2009; Kono *et al.,* 2014; Namikoshi *et al.,* 2004; Witteveldt *et al.,* 2004)，実際の養殖場での普及には程遠い．甲殻類は獲得免疫を持たないため，養殖魚で効果的な不活化ワクチンによる予防は現実的でない．そこで理想的には宿主の生体防御を高めることすなわち免疫の賦活化による予防が望まれる (吉水 2012)．クルマエビ類の免疫賦活については，ペプチドグリカン，5-アミノレブリン酸または乳酸菌の 1 種 *Lactobacillus plantarum* を免疫賦活剤として含有する試料の摂餌により生体防御関連遺伝子転写産物の蓄積量が増加すること，さらに疾病の防除に有効であることなどが報告されている (Ciu *et al.*, 2007; Fagutao *et al.*, 2008; Pedrosa-Gerasmio *et al.*, 2018)．これら研究報告は生体防御関連遺伝子転写量や ATP 活性の上昇などの科学的根拠により，

先に挙げた免疫賦活剤の有効性を評価している．一方，多くのクルマエビ類用免疫賦活剤の研究報告は，感染試験を行いその死亡率のみで疾病への有効性を検討したものが多く，それら免疫賦活剤がどのような仕組みで宿主の生体防御機構を活性化しているのか明らかとしていない．これは，エビ類の生体防御機構に未だ不明な点が多いためであると考えられる．クルマエビ類生体防御機構を詳細に研究することは，科学的な根拠に基づく宿主免疫の賦活化を可能とし，ひいてはクルマエビ類の養殖生産を持続的に発展させるために重要である．

第３節 外部環境に対するクルマエビ類の生体防御機構

水生生物であるクルマエビ類の周囲には環境水が存在する．我々の周囲に存在する空気中には約 10^3 個/m^3 の濃度で微生物が存在するが (外池 1965)，海水中には約 $10^{10\text{-}12}$ 個/m^3 とより多くの微生物が存在する (清水 1992)．すなわちクルマエビ類は常に微生物と隣り合わせで生きている．

クルマエビ類の外部環境との接触器官は外骨格，腸管およびエラが挙げられる．甲殻類の外骨格は，キチンを主成分としたクチクラ層が特徴である．硬い外皮は物質の体内への浸入を防ぎ，体を支えかつ外敵生物から身を護るという利点をもつ (矢野 1977)．一方で，変態および成長の過程で脱皮現象を伴う外皮更新の反復を不可欠とする．外皮更新のプロセスは生体に対して高負荷であり，さらに脱皮後の外皮は柔らかく捕食者 (Lawlor 1976) または病原細菌による感染 (Liu 2004) に曝される危険もある．外骨格は外部からの異物に対しての物理的な防御のみならず生物学的な排除も行っており，カブトガニ *Tachypleus tridentatus* においてビックディフェンシン，タキスタチンあるいはタキプレシン等の抗菌ペプチドが外骨格から検出

されている (朝野 2015; Iijam *et al.,* 2005).

腸管の主機能は摂食物の消化吸収であるが, その際に侵入してきた異物の排除も同時に行う必要がある. 腸管の免疫系は腸管免疫として知られ, ヒトや哺乳類ではそこに存在するリンパ球, パイエル板や粘液層の研究が盛んに行われている (大島・渡辺 2015; 八村 2014). Silveira ら (2018) はバナメイエビの腸管における免疫関連遺伝子を次世代シークエンサーにより網羅的に解析し, 抗菌ペプチドが腸管組織中に浸潤する血球細胞によって産生されること, WSSV やビブリオ属細菌の感染によってそれら抗菌ペプチドの発現量が上昇することを明らかとした. また, 腸管に特異的に発現するレクチンの存在も明らかにされ, クルマエビ類の腸管でもこれらレクチンによる異物の除去が行われていることが示唆されている (Sun *et al.,* 2008; Zhang *et al.,* 2009). さらに, 腸管内の細菌叢を網羅的に解析することでの腸管免疫機構と腸内細菌叢との関連性を探索する試みも行われている (Cornejo-Granados e*t al.,* 2017; Huang *et al.,* 2016).

甲殻類のエラはイオン交換, 体液浸透圧調整, 酸塩基平衡やアンモニア排泄など多くの生理学的プロセスが行われる多機能臓器であるとともに (Henry *et al.,* 2012), WSSV, IHHNV, TSV やビブリオ属細菌の感染標的部位でもある (Alday-Sanz *et al.,* 2002; Clavero-Salas *et al.,* 2007; Lightner 2011). これは, エラが外部環境水と常に接触し病原微生物の侵入経路となりやすいからである. そのため, エラには外部環境水からの異物を排除するための免疫機構が存在する. Amparyup ら (2008) は血球細胞およびエラにおいて発現するクラスチン様抗菌ペプチドの存在, さらに本抗菌ペプチドのグラム陰性および陽性菌に対する強い抗菌活性を明らかにした. この他にもペナエジン (Destoumieux *et al.,* 2000) や Anti-lipopolysaccharide factor (Liu *et al.,*

2005) といった抗菌ペプチドもエラから検出されている．これら抗菌ペプチドは血球細胞から産出されていることが知られており，エラ内部に侵入した細菌に対して働くと考えられている．一方で，Alenton ら (2017) はエラおよび胃に特異的に発現するレクチンの存在および細菌への凝集能を明らかとした．本レクチンはエラ周囲の粘液からも検出されたことから，エラ外部での異物排除に働いていることが示唆された．魚類では体表やエラにはレクチンやムチンからなる粘液が存在し，これらが免疫において大きな役割を果たす (中村・筒井 2016; 村本ら 2003)．エビ類の粘液または粘膜に関する研究報告は少なく，今後の研究が望まれる．

第 4 節 血球細胞が司る生体防御機構

外骨格，腸管およびエラにおける生体防御機構は，いかに外部から体内への異物の侵入を防ぐかというものである．これらを突破して体内に侵入した異物に対して応答するのが血液中に存在する血球細胞である．甲殻類は開放血管系であるため，血液は心臓から動脈を通じて各組織中へ浸潤する形で循環する．クルマエビ類で主要な免疫系として知られる貪食作用，フェノール酸化酵素前駆体活性化系，包囲化，ノジュール化，血液凝固作用，抗菌ペプチド産生やオプソニン化などは血球細胞が中心となり機能している (Jiravanichpaisal *et al.,* 2006; Tassanakajon *et al.,* 2013)．

貪食作用は細胞性免疫であり，外部からの異物を貪食し消化する作用のことである．哺乳類において好中球，マクロファージおよび樹状細胞は，病原体に共通して存在するさまざまな分子構造 (pathogen-associated molecular patterns: PAMPs) を認識し貪食する (Aderem and Underhill 1999)．代表的な PAMPs はグラム陰性菌のリポ多糖，グラム陽性菌のリポテイコ酸および細菌細胞壁の骨格構造であるペプチドグ

リカンである．エビ類においてもリポ多糖およびペプチドグリカンによる刺激が，血球細胞による貪食を促進させる (Itami *et al.,* 1998; Sung *et al.,* 2000; Wang *et al.,* 2017)．さらに，クルマエビ類の貧食細胞は，レクチンによるオプソニン効果により活性化される (Kondo *et al.,* 1998a; Shi *et al.,* 2014; Wang *et al.,* 2017)．真核生物に共通して見られる生体防御機構である貪食作用のメカニズムは種を越えて保存されていると考えられているが，クルマエビ類においてどのようなカスケードの下で異物が貪食されるかその詳細は明らかでない．また，クルマエビ類の貪食作用は血液中に存在する血球細胞のみならず，リンパ様組織および心臓に定着している細胞によっても行われることが Kondo ら (1998b) による組織切片の電子顕微鏡観察および van de Braak ら (2002a) による組織切片の光学顕微鏡観察によって明らかにされている．Kondo ら (1998b) はこれら細胞を固着食細胞と命名したが，その形態は血球細胞と類似しており分子生物学的特徴は明らかでない．

フェノール酸化酵素前駆体活性化系は prophenoloxidase (proPO) 活性化系とも呼ばれ，ペプチドグリカンやβ-1, 3-グルカンにより活性化されるプロテアーゼカスケードであり (芦田 2004; Johansson and Söderhäll 1989)，血液中に存在するフェノール酸化酵素前駆体が活性化されたセリンプロテアーゼによってフェノール酸化酵素となる．フェノール酸化酵素は周囲に存在するチロシンや L-dopa をメラニン化させる．メラニン生成の過程で生じるキノンおよびメラニンは細胞毒性があり，ザリガニではアファノマイセス症の原因真菌である *Aphanomyces* 属細菌に対して毒性を示す (Söderhäll and Ajaxon 1982). クルマエビ類ではこれら一連のカスケードが非自己の認識機構として働き，血球細胞による異物の貪食作用，メラニン化，包囲化およびノジュール形成において重要な働きを示す (Cerenius and Söderhäll

2004; Cerenius *et al.,* 2008; Nappi and Christensen 2005)．さらに，クルマエビにおいて二本鎖 RNA を用いた *in vivo* 遺伝子ノックダウン実験では，フェノール酸化酵素前駆体遺伝子の減少が血中の血球細胞数の減少および血中菌数の増加をもたらし個体の死亡率を上昇させる (Fagutao *et al.,* 2009)．しかし，昆虫・甲殻類を問わずフェノール酸化酵素前駆体活性化系に関連する構成因子がすべて同定されている種はなく，クルマエビ類でも全容の解明に向け研究が進められている．

包囲化は貪食できないような大きな異物を血球細胞が取り囲んで体腔内で隔離し排除する作用である．また小型の異物でも大量に侵入した場合には包囲化と似た機序で血球細胞が集積して異物を包み込みノジュールを形成する．これらの作用は組織や血液中に存在するレクチンがパターン認識受容体として働き，オプソニン化された異物を血球細胞が認識することによって引き起こされる (Junkunlo *et al.,* 2012; Ma *et al.,* 2008; Wang *et al.,* 2013)．包囲化またはノジュール化が起こった部位では，同時にフェノール酸化酵素前駆体活性化系が働きメラニン化も引き起こされる．これら作用は大型または大量の異物を排除する役割のみならず，異物の体内での拡散も防ぐ役割も果たす．しかし，レクチンによってオプソニン化された異物に遊走する際，血球細胞がどのようにして誘引されるかは未だ明らかになっていない．レクチンそのものが誘引物質となっている可能性もあるが，今後の詳細な研究が望まれている (Burge *et al.,* 2007)．

甲殻類の血液凝固作用のカスケードは，ザリガニおよびアメリカカブトガニ *Limulus polyphemus* で詳細な研究が進められてきた．血液凝固カスケードは，凝固タンパク質 (clotting protein: CP) がカルシウム依存性のトランスグルタミナーゼ (transglutaminase: TGase) の働きで凝固することにより引き起こされる一連の反応

系である．ザリガニでは血球細胞や他の組織から放出される TGase によって CP の凝固が起こることで血液が凝固する (Hall *et al.,* 1999; Kopácek *et al.,* 1993; Sritunyalucksana and Söderhäll 2000; Wang *et al.,* 2001)．一方，アメリカカブトガニの血液凝固カスケードでは TGase の働きによる CP の凝固によって血液凝固反応が起こるのみならず，抗菌ペプチドの発現も促進される (Iwanaga 1993; Muta and Iwanaga 1996)．これら抗菌ペプチドは凝固部位にて共同で作用し，細菌の排除を行う (Iwanaga and Lee 2005)．アメリカカブトガニの血液凝固カスケードはグラム陰性菌と強く反応し凝固するため，医薬品への細菌の混入検出試薬 Limulus amebocyte lysate (LAL) として広く用いられている．クルマエビ類の血液凝固カスケードはカブトガニと類似しており，TGase の作用により CP が凝固するのみならず，TGase が血球細胞からの抗菌ペプチドの発現を司っている (Fagutao *et al.*, 2009; Maningas *et al.*, 2013)．また，α2-macroglobulin と呼ばれるタンパク質が TGase の基質として働き CP と共に血液凝固を引き起こす (Chaikeeratisak *et al.,* 2012; Ponprateep *et al.,* 2017)．さらにクルマエビでは，TGase および CP を標的として二本鎖 RNA による *in vivo* 遺伝子ノックダウンを行うと，WSSV および *Vibrio penaecida* 感染に対する抵抗性が弱まること，また，いずれの病原菌を感染させなくとも個体が死亡する (Maningas *et al.,* 2008).

抗菌ペプチドは病原性細菌から宿主を守るための液性免疫の一つであり，細菌から無脊椎動物，脊椎動物，植物にわたり広い生物種に存在する 15 から 100 アミノ酸残基で構成されるタンパク質である (岩室 2009)．抗菌ペプチドの活性はそのアミノ酸組成と配列に基づく立体構造に大きく依存し，細菌類や真菌類あるいは一部のウィルスなどに対して広範囲な抗菌スペクトラムを示す (Boman 1995; Ganz and

Lehrer 1999; Zasloff 2002)．クルマエビ類でも多くの抗菌ペプチドの報告があり代表的なものとしてペナエジン，クラスチンおよび抗リポ多糖類因子が挙げられる (Tassanakajon *et al.,* 2010)．抗菌ペプチドは体内へ侵入した細菌に対して速やかに働くため, 体内での細菌拡散および増殖を防ぐ役割も果たすため開放血管系であるクルマエビ類において最も重要な生体防御関連遺伝子群の一つであると考えられる．さらに，ブラックタイガーのペナエジンはサイトカイン様の機能を有し，血球細胞の遊走を誘導する (Li *et al.,* 2010)．このように，抗菌ペプチドはただ単に細菌を溶菌するのみではなく多様な働きをすることがわかってきた．

これら機能を詳細に解析することが, 血球細胞の司る生体防御機構全体を包括的に理解することにつながる. 細胞機能を詳細に解析するためにはその機能を有する細胞集団を分離し，研究を行うことが有効である．しかしながら，いずれの血球細胞集団がいずれの細胞機能を有するのかが明確になっていない. その理由はクルマエビ類血球細胞のサンプリング手法や分類手法が統一されていないためである. そのため研究室または研究者間で得られる結果に齟齬が生じ, クルマエビ類血球細胞研究の発展を妨げているのが現状である. そのため血球細胞の分類体系を確立することはクルマエビ類の生体防御機構を理解するために重要な基礎研究であることは疑いようもない．

第５節　クルマエビ類血球細胞分類とその機能

甲殻類の血球細胞は Bauchau (1981) 以来，無顆粒球 (Hyaline cells: HCs)，小顆粒球または半顆粒球 (semi-granular cells: SGCs)，大顆粒球または顆粒球 (Granular cells: GCs) の 3 種類に，形態に基づき分類することが一般的である．血球細胞の形

態観察には，一般的にギムザ染色を施された標本が用いられる．

ギムザ染色法は 1904 年にドイツの細菌学者である Gustav Giemsa によってマラリア原虫の染色法として開発された．ギムザ染色法は，pH: 6.3-7.3 の緩衝液中で青色の塩基性色素 (メチレン青・アズール青) と赤橙色の酸性色素 (エオジン) が混在することで, 単に青色や赤橙色のみでなく多種の色調が得られる Romanowsky 効果を基調とした染色法である．アズール青は，好塩基性物質 (核の DNA，細胞質の RNA，アズール顆粒など) を青紫色に染める．エオジンは，好酸性物質 (ヘモグロビン，好酸性顆粒など) を赤橙色に染める．血球の中の物質および構造はこの両色素に染色されて，血球細胞毎に特徴的な染色像を呈する．

クルマエビ類の血球細胞も形態に基づき 3 種類に分類されるが (近藤ら 1992; Kondo *et al.,* 1998b)，ギムザ染色法の改法であるメイグリュンワルド-ギムザ染色法 (メイギムザ法) により 8 種類に分類される報告もある (近藤ら 2012)．同様にバナメイエビではイオジキサノール密度勾配遠心法により 5 種類 (Dantas-Lima *et al.,* 2013)，ブラックタイガーでは電子顕微鏡観察により 4 種類 (van de Braak *et al.,* 2002b) と研究者によってはさらに細分化した分類の報告もある．

細胞機能と形態的分類との比較検討も行われている．近藤ら (1992) はクルマエビの血球細胞を Percoll 密度勾配遠心法にて HCs，SGCs および GCs の 3 種類に分離した．これら血球細胞の貪食活性を調べたところ，いずれの血球細胞もグルタルアルデヒド固定ヒツジ赤血球を貪食し，SGCs と GCs の貪食率が HCs よりも 2 から 3 倍高かったことを報告している．また，血球細胞による固定ヒツジ赤血球の貪食率が, クルマエビ血清によるオプソニン化により著しく増大することを報告している (近藤ら, 1992)．同じクルマエビ類であるバナメイエビではイオジキサノール

密度勾配遠心法にて分類される 5 つの亜集団の内，亜集団 1 および 4 が貪食活性を有すると Tuan ら (2016) により報告されている．オニテナガエビ *Macrobrachium rosenbergii* では GCs および SGCs によるザイモザンの貪食が確認されている (Sung *et al.,* 2000)．また，ザリガニでは HCs および一部 SGCs が貪食活性を有する (Johansson *et al.,* 2000)．ヨーロッパミドリガニ *Carcinus maenas* では HCs が，アメリカンロブスター*Homarus americanu* では GCs および SGCs がそれぞれ貪食活性を有する (Hose *et al.,* 1990)．以上のように，貪食を行う血球細胞とその形態的分類は研究者または種によって結果が大きく異なる．フェノール酸化酵素前駆体活性化系と血球細胞分類に関する研究も行われ，こちらは顆粒を有する血球細胞にフェノール酸化酵素前駆体活性化系関連遺伝子の転写産物および関連酵素が蓄積されている (Söderhäll 2016; Sung *et al.,* 1998; Yang *et al.,* 2015)．また，ペナエジン，クラスチンおよび抗リポ多糖類因子といった抗菌ペプチドは血球細胞の顆粒中に蓄積されている (Bachère *et al.,* 2004; Rosa and Barracco 2010)．

フローサイトメーターを用いて血球細胞の形態と細胞機能を検討する論文も多数報告されている．フローサイトメーターは短時間に多くの細胞を客観的かつ同一条件で測定可能である．Sun ら (2010) や Yang ら (2015) はフローサイトメーターにて血球細胞の形態を解析すると共に細胞の分取も行いその細胞機能や転写産物を解析している．しかしながら，多くの論文で解析を行う領域の細胞分取を行わず，展開図中の相対的な値のみから細胞の分類を行っており，領域中の分類された細胞が本当に目的の細胞であるのか不明のまま議論がなされている (Du *et al.,* 2010; Lee *et al.,* 2000; Sequeira *et al.,* 1995; Xian *et al.,* 2013; Xian *et al.,* 2018)．これら研究報告は研究室または研究者間での議論に齟齬を生じ，血球細胞研究の発展を妨げている

一つの要因にもなっている．

ヒトの白血球はギムザ染色法により，好中球，好酸球，好塩基球，リンパ球および単球の 5 種類に大別される．しかしながらギムザ染色法の技法は統一されておらず，希釈に用いるバッファーの pH，染色時間，染色時の湿度や作業者の修練度などの様々な要因によりその染色結果は容易に変化する．そこで，1975 年に Köhler と Milstein が開発したモノクローナル抗体を用いて形態的分類に加え分子生物学的にも分類することがヒト白血球分類では主流である．ヒトの白血球を中心とした細胞表面に存在する分子に対するモノクローナル抗体を用いた分類手法は cluster of differentiation (CD) 分類と呼ばれる．これら標的となる分子は，モノクローナル抗体が結合する抗原であり，表面抗原あるいは表面マーカーと呼ばれる．異なる種類のモノクローナル抗体が同じ表面抗原分子に結合することがあるため，同一表面抗原分子を認識する抗体群を同じ番号で国際的に統一して分類したものが CD 分類である．CD 分類はモノクローナル抗体の分類であるが，モノクローナル抗体が認識する表面抗原分子の名称にも用いられ，例としてヘルパーT 細胞およびキラーT 細胞のマーカーとして知られる CD4 や CD8 が挙げられる．

クルマエビ類血球細胞の形態もヒトの白血球同様にギムザ染色のみでは判別困難な場合が多い．これまでにクルマエビ類の血球細胞に対して，モノクローナル抗体を作製し分類を試みた研究報告がある．Rodriguez ら (1995) はクルマエビ全血球細胞を抗原とし，10 種類のモノクローナル抗体を作製した．Sung ら (1999) および Sung and Sun (2002) はブラックタイガーの全血球細胞を抗原とし，4 種類のモノクローナル抗体を作製した．同様に van de Braak ら (2000) や Winotaphan ら (2005) もブラックタイガーの全血球細胞や血球細胞溶解物を抗原としてそれぞれ

4 種類のモノクローナル抗体を作製した．これらモノクローナル抗体を用いた免疫学的染色の結果，GCs，SGCs および HCs といった形態的分類上同一の細胞でもその細胞表面抗原に対する反応性に相違が見られ, モノクローナル抗体の反応性の差異によって血球細胞がより詳細に識別されることが指摘されている．さらに，Lin ら (2007) および Zhan ら (2008) によって作製されたバナメイエビ血球細胞に反応を示すモノクローナル抗体を使用し, Xing ら (2017) が全血球細胞を 2 つの亜集団，無顆粒球集団および顆粒球集団に分離することに成功している．同グループはそれら亜集団のプロテオーム解析を行い細胞機能の推定をしている (Zhu *et al.,* 2018a; Zhu *et al.,* 2018b)．しかしながら，これらモノクローナル抗体を用いた手法がスタンダードとはなっていない．考えうる理由として，全く同一のモノクローナル抗体産生クローンは他研究室では調製できないことや研究者の数が少ないため製品として取り扱う企業が存在しないことが考えられる．

そこで，抗体に頼らず特定の細胞を分類することも重要である．哺乳類，特にヒトでは細胞表面に存在する糖鎖に基づいた細胞の分類が行われている (Christiansen *et al.*, 2014; Gabius *et al.*, 2015)．レクチンは糖鎖に結合するタンパク質の総称であり, 糖鎖に対する特異性から血球細胞の染色や分類にも利用されている．これまでにミツバチ *Apis mellifera*，ショウジョウバエ *Drosophila melanogaster*，ガンビエハマダラカ *Anopheles gambiae*，マガキ *Crassostrea gigas* およびヨーロッパイガイ *Mytilus edulis* の血球細胞がレクチンによって染色されてきた (Jiang *et al.,* 2016; Marringa *et al.,* 2014; Pipe 1990; Rodrigues *et al.,* 2010; Tirouvanziam *et al.,* 2004)．また，Martin ら (2003) によりイシエビ属の 1 種 *S. ingentis* およびアメリカンロブスター*H. americanus* の血球細胞の細胞質内顆粒が小麦胚細胞凝集素 (wheat-germ

agglutinin: WGA) により特異的に染色されることが報告されている．さらに，Estrada ら (2016) によりバナメイエビの血球細胞 GCs，SGCs および HCs に対して WGA，トマト凝集素 (*Lycopersicon esculentum* agglutinin: LEA) およびピーナッツ凝集素 (peanut agglutinin: PNA) がそれぞれ一定の割合で結合することが報告されている．しかしながら，クルマエビ類血球細胞のレクチンによる染色は未だ前述のバナメイエビに対する報告のみであり少なく，レクチンにより分類される各特異的血球細胞集団の分取もなされていない．

第 6 節 クルマエビ類からの採血時に用いられる抗凝固液および固定液について

クルマエビ類から採血を行い，血液学的性状試験を行う際や血球細胞を分離する際には抗凝固液が用いられる．前述の通りクルマエビ類の血球細胞には様々な免疫作用が存在するが，血液学的性状試験および血球細胞の研究を行う際に，フェノール酸化酵素前駆体活性化系および凝固作用を阻害する必要がある．現在最も使用されている甲殻類血液研究のための抗凝固液は Söderhäll and Smith (1983) によって発表されたものである．その組成は，100 mM Glucose，30 mM trisodium citrate，26 mM citric acid，510 mM NaCl，10 mM EDTA-Na2，pH: 4.6 である．本抗凝固液は Alsever 液と呼ばれるヒトの全血を保存する際に用いられる溶液 (114 mM Glucose，30 mM trisodium citrate，2.65 mM citric acid，71 mM NaCl，pH: 6.5) を甲殻類用に主に浸透圧を調整したものである．クルマエビ類を含む甲殻類の血液中のフェノール酸化酵素前駆体活性化系や凝固作用は，二価の陽イオン特にカルシウムイオンに依存している (Gollas-Galván *et al.,* 1997; Maningas *et al.,* 2013)．本抗凝固液には，クエン酸カルシウムを生じることにより血中のカルシウムを除去する trisodium citrate

および二価の陽イオンをキレート化する EDTA が含まれており血液中のフェノール酸化酵素前駆体活性および凝固作用を抑える働きがある. 本抗凝固液の原法では pH: 4.6 で用いることを推奨しているが，クルマエビ類では採血後にタンパク質の析出が起こることが報告されており，pH: 5.6-7.0 で用いられることが一般的である (Vargas-Albores *et al.*, 1993).

次に使用が多いものは, Vargas-Albores ら (1993) によって報告された shrimp salt solution (SSS) である．その組成は，450 mM NaCl，10 mM KCl，10 mM EDTA, Na2，10 mM HEPES，pH: 7.3 である．前述の抗凝固液同様に，EDTA がキレート剤となり二価の陽イオンの働きを阻害することで抗凝固液としての役割を果たす. Vargas-Albores らは SSS の調整に際して，*Penaeus stylirostris* の血液浸透圧，組成や塩濃度を調査した (Vargas-Albores *et al*, 1992; Vargas-Albores and Ochoa, 1992). そのため SSS は前出の Söderhäll and Smith の抗凝固液と比較し，クルマエビ類血液の生理的条件により近い抗凝固液といえる. いずれの抗凝固液も血液の凝固およびフェノール酸化酵素前駆体活性化を阻害し, フェノール酸化酵素前駆体活性化系の機能解析実験を行うことに成功しているため, これら抗凝固液を用いることが血液の生化学試験および血球細胞の分離を行う際に重要である.

血球細胞の研究，特に血球細胞に免疫学的染色を行う際には，血球細胞を固定処理することが一般的である．Wang ら (2002) および Sun and Sung (2002) は，採血をする際に 10%ホルマリン溶液を用いて直接血球細胞の固定を行っている．同様に, 筆者の所属する研究室でも抗凝固液で採血した後の血球細胞に対して免疫学的染色を施す際に，ホルマリンによる固定を行っている (Elbahnaswy *et al.,* 2017; Zhang *et al.*, 2018a; Zhang *et al.*, 2018b)．これはクルマエビ類の血球細胞が未固定の

状態では物理的な刺激に弱く遠心操作によるペレット化やピペッティングなどの操作により容易に細胞が損傷または損壊してしまうからである. 溶液中に懸濁状態の未固定クルマエビ血球細胞が抗体により分類された研究報告はない. 最も一般的な固定液はホルマリンであるが, ホルマリンは細胞中の核酸を他の成分と共有結合させ，その抽出等を困難にする (Coombs *et al.*, 1999; Masuda *et al.*, 1999). そのため採血および固定処理を行った血球細胞を抗体やセルソーターなどにより分類および分離しても，それら細胞から各種遺伝子工学的実験に用いるための RNA を抽出することは非常に困難である．近年，dithiobis [succinimidyl propionate] (DSP) によりアミノ基を架橋することで，細胞固定およびその後の RNA 抽出が可能になるとの報告がある (Attar *et al.*, 2018; Xiang *et al.*, 2004). DSP を利用した研究報告は少なく，クルマエビ類血球細胞に適用された例はないが，このような新規の固定剤を用いることも今後の血球細胞研究には重要である.

生細胞の形態は外部の浸透圧の変化により容易に変化する. 甲殻類の血球細胞も例外ではなく, そのため各種類のエビに対して適切な浸透圧の抗凝固液または固定液を用いることは重要である. 脊椎動物の血液浸透圧は恒常性が維持され一定であるのに対して，甲殻類の血液浸透圧は外部の環境水に併せて変動する．そのためクルマエビの血液浸透圧は約 680-1050 mOsm/kg (Setiarto *et al.*, 2004)，バナメイエビでは約 480-800 mOsm/kg (Rosas *et al.*, 2002; Sowers *et al.*, 2006)，ブラックタイガーでは約 680-760 mOsm/kg (Chen et al., 1986; Owens and Smith, 1999) と，環境水の塩濃度によりその血液浸透圧も変動する. そのため多くのエビ用抗凝固液もしくは固定液では，浸透圧が 800-1100 mOsm/kg 程度に調製されている．ヒト血液浸透圧の基準値は 275-290 mOsm/kg で有ることを考えると, 海産甲殻類の血液浸透圧がいか

に高浸透圧であり，その変動の幅が大きいかが見て取れる．

以上のようにクルマエビ類の血液生化学試験および血球細胞分離の際に用いられる溶液は，いくつか報告されている．異なる溶液を用いると，血液性状および血球細胞の形態は変化する．そのため目的に沿って適切な抗凝固液および固定液を用いることが重要である．また，市販の研究用溶液やキットの多くは，哺乳類を対象に開発が進められているため，溶液の浸透圧条件なども哺乳類のものに適して作製されている．そのため試薬やキットの組成や生理的条件を確認し，必要がある場合には塩を加えるなどして浸透圧条件を適切にすることが重要であることをここに改めて記載する．

第７節　本研究の目標

クルマエビ類の血球細胞が司る生体防御研究は，発現する遺伝子のクローニングおよび発現変動解析を中心に研究が進められ，主要な免疫系が明らかになってきた．しかしながら，これまでの研究では全血球細胞を材料として研究が行われたため，いずれの血球細胞集団がいずれの免疫機能を有するのかが明確になっていない．免疫系を細胞集団レベルで研究するために，血球細胞の分類手法を開発することは極めて重要である．また，分子マーカーを指標に血球細胞を分類できるということは，病原微生物の感染や免疫賦活剤投与時における血球細胞集団の変動を解析することを可能とし，クルマエビ類の生体防御応答を詳細に解析できることが期待される．さらに，客観的な血球細胞の分類手法の開発は，研究者および研究室間での統一した研究結果を得ることに繋がり，これまでにもましてクルマエビ類生体防御研究の議論を活発にさせる．

そこで本研究ではクルマエビ血球細胞の客観的な分類および分離手法開発のための研究を行った．第 2 章では，組織中に定着している血球細胞機能の分子生物学的機能解析を，次世代シークエンサーによる網羅的転写産物解析により行った．第 3 章では，貪食能を有する血球細胞特異的細胞表面分子を探索するため磁気マイクロビーズを用いた貪食細胞の濃縮手法を開発し，濃縮された貪食細胞で特異的に蓄積される遺伝子転写産物を同定し抗体を用いた免疫学的染色により本遺伝子が貪食細胞のマーカーとなり得るかを評価した．第 4 章では，血球細胞を糖鎖に基づき分類するため血球細胞の亜集団に特異的に結合するレクチンの探索を行い，結合を示したレクチンおよび細胞磁気標識手法を利用し，血球細胞の亜集団を分離し転写産物を解析することで機能を推察した．

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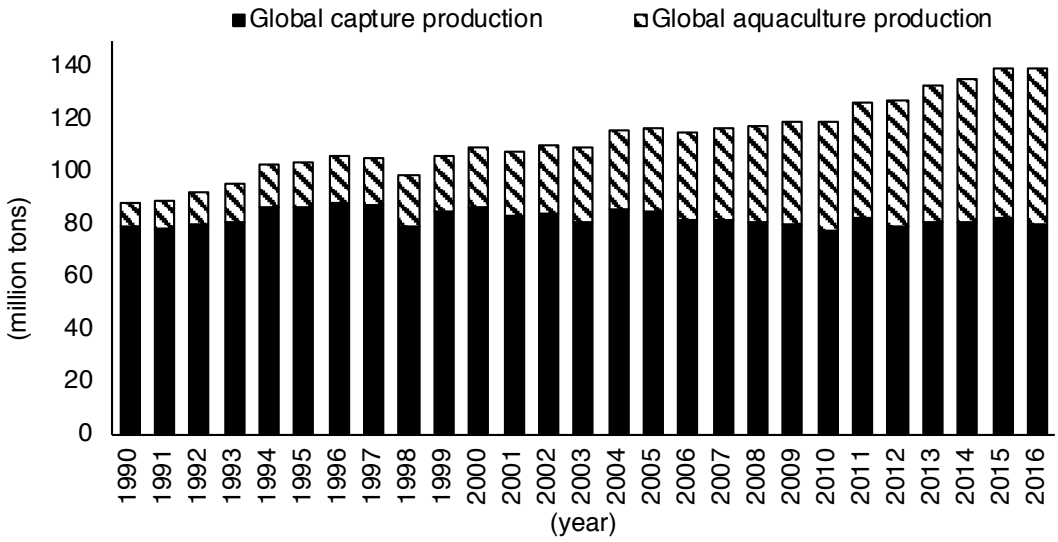

図 1. Global production of fisheries from capture and aquaculture. Data was collected from The FAO Fisheries and Aquaculture Department Statics, http://www.fao.org/fishery/statistics/en, accessed 2018-10-30.

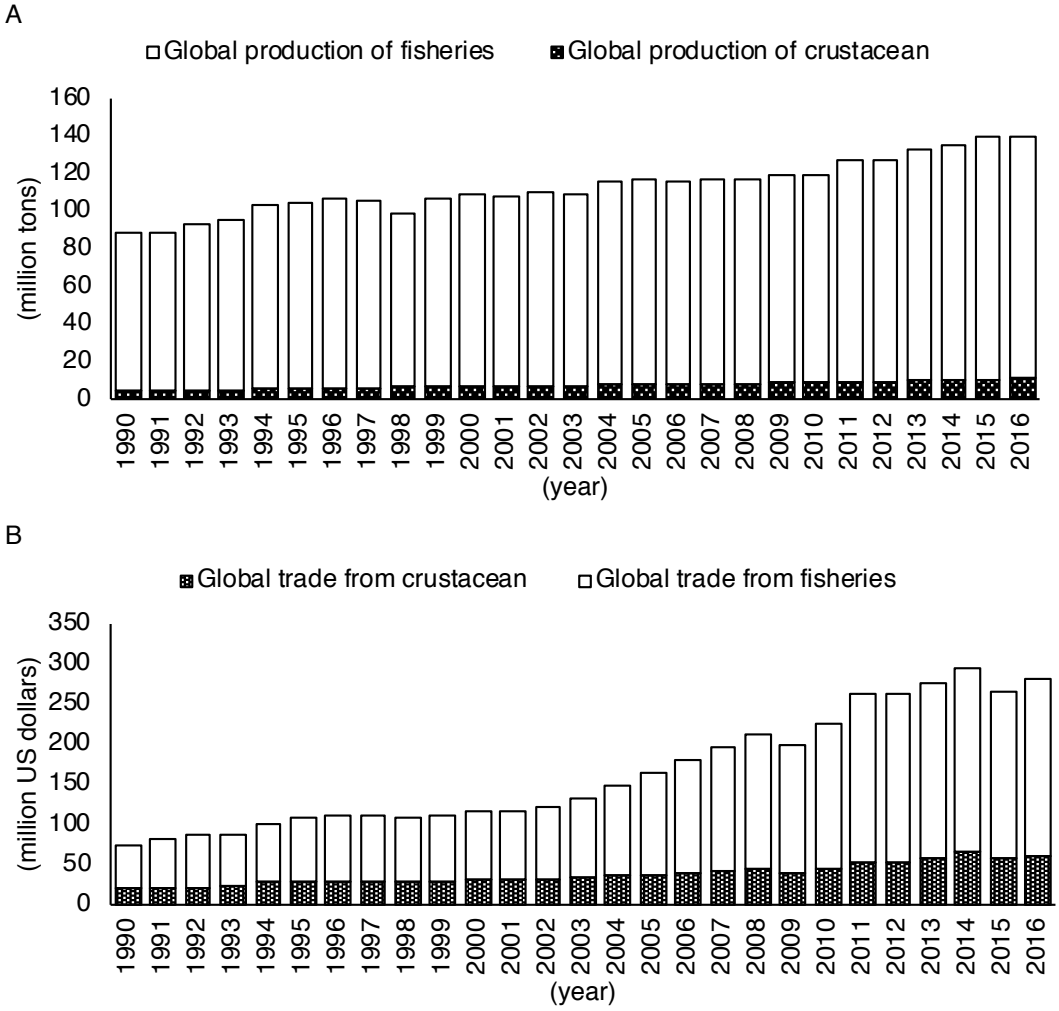

図 2. Global production and trade of crustacean. Global production of crustacean (A) and global trade of crustacean (B). Data was collected from The FAO Fisheries and Aquaculture Department Statics, http://www.fao.org/fishery/statistics/en, accessed 2018-10-30.

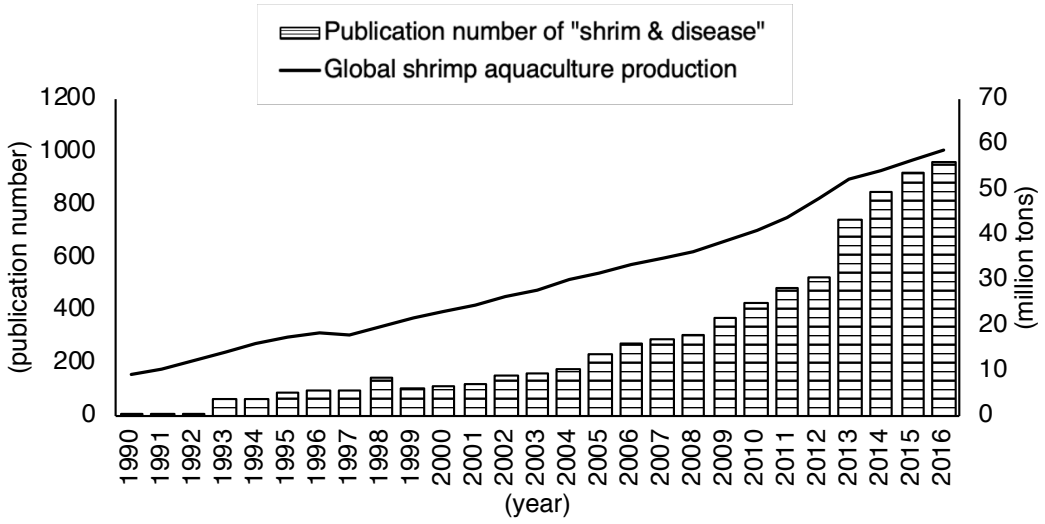

図 3. Correlation diagram between the number of publications of “shrimp & disease” and global production of crustacean. Data was collected from The FAO Fisheries and Aquaculture Department Statics, http://www.fao.org/fishery/statistics/en, accessed 2018-10-30, and Web of Science, http://www.webofknowledge.com/wos, accessed 2018-10-30.

第２章　The immune functions of sessile hemocytes in three organs of kuruma shrimp *Marsupenaeus japonicus* differ from those of circulating hemocytes

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第１節 Abstract

Shrimp, as invertebrates, have an open vasculature that allows circulating hemocytes to infiltrate the tissues, where they are referred to as sessile hemocytes. Sessile hemocytes are known to express immune-related genes, but it is not known whether their functions differ from those of circulating hemocytes. To answer this question, I enriched them from suspensions of different tissues using discontinuous density gradient centrifugation and analyzed their transcripts by RNA-seq. The results suggest that circulating hemocytes and sessile hemocytes of the gills are in a state that could react quickly to pathogens, immune-related genes expression of sessile hemocytes differ from circulating hemocytes, and the gills, heart and lymphoid organs have cells that express immune-related genes that are different from hemocytes.

第２節 Introduction

In invertebrates, hemocytes have roles in several immune-related processes such as phagocytosis, coagulation, encapsulation, nodulation, production antimicrobial peptides (AMPs) and prophenoloxidase (proPO) activity (Jiravanichpaisal *et al.,* 2006; Tassanakajon *et al.,* 2013). Invertebrates have open vasculatures that allow circulating hemocytes to infiltrate the tissues, where they are referred to as sessile hemocytes. So far, most studies of hemocyte function have focused on circulating hemocytes, while little is known about the functions of sessile hemocytes. The functions of immune-related genes in different tissues have been examined by expressed sequence tag (Gross *et al.,* 2001; Tassanakajon *et al.,* 2006) and microarray (Aoki *et al.,* 2011; Robalino *et al.,* 2007) analyses. Such studies have shown that immune-related genes are expressed in circulating hemocytes as well as other tissues. However, each of these experiments extracted RNA from whole tissues, so that it is not known whether the source of these immune-related genes is sessile hemocytes or other cell groups.

Wang *et al.* (2007) examined the expression of nine immune-related genes in nine tissues of *Litopenaeus vannamei* using RT-PCR, qRT-PCR, and *in situ* hybridization. They found that sessile hemocytes express several immune-related genes, such as proPO, transglutaminase (TGase), crustin, penaeidin-3, lysozyme and cytosolic manganese superoxide dismutase (cMnSOD). However, because their methods targeted only a limited number of genes, other important genes may have been overlooked.

Distinct cells can be extracted from a tissue suspension by several methods, such as density gradient centrifugation, flow cytometry, and magnetic antibody separation. Density

gradient centrifugation is often easier and less expensive than the other methods. Previous studies have fractionated penaeid shrimp hemocytes by Percoll (Havanapan *et al.,* 2016; Itami *et al.,* 1999; Koiwai *et al.,* 2017; Rodriguez *et al.,* 1995) and iodixanol (Dantas-Lima *et al.,* 2013) density gradient centrifugation and determined their specific gravity.

Here, I developed a technique for concentrating sessile hemocytes from tissue suspensions using discontinuous density gradient centrifugation, and attempted to clarify their function by RNA-seq analysis.

第３節 Material and methods

Shrimp samples

Apparently healthy kuruma shrimp (*Marsupenaeus japonicus*), obtained from farms in Okinawa and Miyazaki prefecture, Japan, were used in this study. Shrimps were kept and acclimatized for at least 3 days before the experiment in tanks provided with recirculating water maintained at 25 ^{0}C and 30-35 ppt.

Density gradient centrifugation

Single-cell suspensions from gills, heart, and lymphoid organs were obtained using a 40 μm nylon cell strainer (Corning Inc., USA) from a shrimp, diluted with kuruma shrimp PBS (KPBS) (480mM NaCl, 2.7mM KCl, 8.1mM $Na_2HPO_4 \cdot 12H_2O$, 1.47mM KH_2PO_4, pH: 7.4), placed onto 1.05/1.11 g/mL discontinuous Percoll (GE Healthcare, USA) density gradients, and centrifuged at 500 xg for 20 min. Cells at the interface were collected and washed twice in washing buffer (KPBS containing 0.1% of BSA and 10 mM EDTA), and was likewise performed for Giemsa staining, flow cytometry analysis and cDNA library construction.

Flow cytometry analysis of sessile hemocytes

Sessile hemocytes were obtained in the same way as described in above section, then fixed with fixing buffer (KPBS containing 10% formalin and 10 mM EDTA). Both non-concentrated and concentrated cell suspension were analyzed by flow cytometry. Forward-scatter (FSC) and side-scatter (SSC) were used to determine relative cell size and relative cell complexity, respectively. Five thousand (5,000) events of each sample were collected

and FSC and SSC analyses were conducted by FACSCalibur (Becton-Dickinson, USA) with Cell Quest Pro software ver. 5.2.1 (Becton-Dickinson). Two gates, debris and hemocytes, were established based on the FSC and SSC, and then the percentage of dot plots in each gate were analyzed by Cell Quest Pro software. The assay was performed three times.

Giemsa staining

Circulating hemocytes were collected using anti-coagulant buffer (KPBS containing 10 mM EDTA) and washed twice in washing buffer, then fixed with fixing buffer. Sessile hemocytes were collected and fixed from three shrimps in the same way as described in above section. Each fixed cell suspension was spread on glass slide using cell collection bucket SC-2 (TOMY, Japan) at 10 xg for 2 min. Glass slides were dried, fixed again with ice-cold methanol for 3min, dried again, stained for 20 min with 5% Giemsa stain solution (Wako, Japan) in 0.067 M phosphate buffer (pH: 6.6), washed with tap water, dried, mounted with Malinol (Muto Pure Chemicals, Japan) and visualized with NIS-Elements software (Nikon, Japan). The assay was performed three times.

cDNA library construction and sequencing by Illumina Miseq

Circulating hemocytes, tissue of gills, heart, lymphoid organs, and sessile hemocytes of those tissues were collected from three shrimps in the same way of above sections. Total RNA was extracted using NucleoSpin® RNA XS (Takara Bio Inc., Japan) following the manufacturer's protocol. The concentration and purity of total RNA were using a Qubit® RNA BR Assay Kit and NanoDrop Lite (both Thermo Fisher Scientific Inc., USA). cDNA

libraries were prepared using TruSeq stranded mRNA sample preparation kit (Illumina Inc., USA) followed the manufacture's protocol. The libraries were amplified with 15 cycles of PCR and contained indexes within the adaptors. The yields in the amplified libraries were measured with a Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific Inc.) and D1000 ScreenTape System (Agilent Technologies, USA). The libraries were diluted to 10 nM and sequenced using MiSeq (Illumina Inc.) and MiSeq reagent kit version 2 (Illumina Inc., USA) with 150 nt paired end reads.

De novo assembly, Trinity-assembled gene quantification, and quality check of samples and biological replicates

The reads were assembled by Trinity software v2.5.1 (Grabherr *et al.,* 2011) using default parameters (minimum assembled transcripts length 200) to obtain Trinity-assembled genes, running with Trimmomatic to perform quality trimming using default parameters. Each sequenced library was mapped back to the reference Trinity-assembled genes using RSEM (Li and Dewey, 2011) to quantify the transcripts per kilobase million (TPM) value. TPM value were normalized by trimmed mean of M-values (TMM) as TMM-TPM value to account for differences in library size (Robinson *et al.,* 2010), then log2-transformed as Log TMM-TPM value. Trinity-assembled gene quantification for each of the samples and biological replicates was examined by correlation matrix and principal component analysis (PCA) using a Perl-to-R script included in Trinity.

Homology searching and identification of immune-related Trinity-genes

A search of the NCBI database for proteins using "Penaeidae" as the organism yielded 5,299 proteins as of Nov. 11, 2017. This database was then searched for homologs of each of Trinity-genes using Blastx (Altschul *et al.,* 1997). To comprehensively characterize immune related genes, Trinity-genes which log TMM-TPM value was higher than 8.0 were extracted. The log TMM-TPM value of housekeeping Trinity-genes were around 12.0, thus log TMM-TPM value 8.0 indicates 1/16 expression against housekeeping genes. Among the homologs found in the Penaeidae protein database, I searched for immune-related genes, in particular genes involved with AMPs, clotting, proPO and lectin. Heat maps of these genes were constructed with MeV (https://github.com/dfci-cccb/mev).

第 4 節 Results and discussion

Previous studies reported that hemocytes had a specific gravity in the range 1.05-1.10 g/mL (Dantas-Lima *et al.,* 2013; Koiwai *et al.,* 2017; Rodriguez *et al.,* 1995), so I used 1.05 and 1.11 g/mL Percoll layers in the present study. As expected, hemocyte-bearing tissues yielded a layer of cells between these layers. These cells were confirmed to be hemocytes by Giemsa staining. (Fig. 1). Many of the hemocytes from each tissue contained granules in their cytoplasm (Fig. 1). As shown by the flow cytometry analysis (Fig. 2), sessile hemocytes were enriched many times by density gradient centrifugation: from 6.2 ± 2.0% to 37.0 ± 26.5% in the gills, from 2.9 ± 0.2% to 22.0 ± 16.2% in the heart and from 3.5 ± 0.3% to 18.6 ± 9.5% in the lymphoid organs.

The Trinity software assembled the transcripts into 67,284 genes, referred to below as Trinity-genes, with a median length of 367 bp and a weighted median N50 of 1,446 bp. Both correlation matrix (Fig. 3) and PCA (Fig. 4) result indicated the replicates are highly correlated within same tissue. This shows that the comparative analysis based on the results obtained from RNA-seq analysis was appropriate.

Of the 67,284 Trinity-genes, 43,900 had hits in the Penaeidae protein database with E-values less than 10 (The data is stored in the laboratory). After genes with relatively low expression levels were removed, 874 genes were left (The data is stored in the laboratory). Of these 106 Trinity-genes, which accounted for 12% of relatively high expression levels of Trinity-genes, were immune-related genes contained 27 AMPs-related, 23 clotting-related, 28 proPO-related, and 28 lectin-related (Table 1).

AMPs-related Trinity-genes were mainly expressed in circulating hemocytes (Fig. 5A).

Several of the genes, marked ch, were expressed only in circulating hemocytes, while genes marked gt were expressed only in gill tissue. Crustin was the top Blastx hit of several of these AMPs-related Trinity-genes (Table 1). Crustin is an AMP unique to the Crustacea (Tassanakajon *et al.,* 2013) and is known to be expressed by hemocytes (Rattanachai *et al.,* 2004). Three crustin-related Trinity-genes were expressed in sessile hemocytes of gills, marked gh. These results suggest that the gills have crustin-expressing cells, and that the expression pattern of AMPS in sessile hemocytes of the gills is different the expression pattern of crustin in circulating hemocytes.

Clotting- and proPO-related Trinity-genes were strongly expressed in circulating hemocytes and sessile hemocytes of gills. On the other hand, these Trinity-genes were not expressed in sessile hemocytes and tissues of heart and lymphoid organ (Fig. 5B, C). The clotting system is considered the first line of defense of the invertebrate immune system (Maningas *et al.,* 2013). The ProPO system also serves an important role as a non-self-recognition system that participates in the innate immune responses (Amparyup *et al.,* 2013). The circulating hemocytes and gills are primary cell or tissues that face to pathogens because of more exposure chances to the external environment than the heart and lymphoid organs, and have a high probability of contact with various bacteria. Because these hemocytes need to be ready to react quickly to pathogens, the expression patterns of their clotting- and proPO-related Trinity-genes would be expected to be different from those of the sessile hemocytes of heart and lymphoid organs.

On the other hand, lectin-related Trinity-genes (Fig. 5D) were expressed in the gills, heart and lymphoid organs as well as circulating hemocytes. Lectins have roles in many processes,

including protein trafficking, cell signaling, and pathogen recognition, through their capacity to bind carbohydrates (Wang and Wang, 2013). Some lectins are expressed by circulating hemocytes (Liu *et al.,* 2007; Wang and Wang, 2013), while others are expressed by specific tissues (Alenton *et al.,* 2017; Sun *et al.,* 2008; Wang and Wang, 2013). Some lectin-related Trinity-genes were expressed more strongly in the gills, heart and lymphoid organs than in the sessile hemocytes of those tissues (Fig. 5D). In Chinese shrimp, a C-type lectin was expressed in the central antrum of the hepatopancreatic duct but not by sessile hemocytes in this tissue (Sun *et al.,* 2008), as well as in *M. japonicus*, another C-type lectin was not expressed by circulating hemocytes (Alenton *et al.,* 2017), indicating that some lectins are also expressed by cells other than hemocytes. Two Trinity-genes, DN29044_c3_g2 and DN29665_c2_g2, were expressed more strongly by sessile hemocytes of the gills, heart and lymphoid organs than their tissues, suggesting that these hemocytes differ from circulating hemocytes.

The above results suggest that 1) circulating hemocytes and sessile hemocytes of the gills, unlike sessile hemocytes of the heart and lymphoid organs, are in a state that could react quickly to pathogens, 2) sessile hemocytes of the gills, heart and lymphoid organs differ from circulating hemocytes, and 3) the gills, heart and lymphoid organs have cells that express immune-related genes that are different from hemocytes.

Further studies are needed to understand how and where hemocytes are produced and how they mature. One approach to answering these questions is to develop molecular markers in crustaceans (Söderhäll, 2013; Söderhäll, 2016). In the present study, I was able to separate only a few sessile hemocytes from hematopoietic tissues because these tissues were very

small. Thus, it was difficult to completely separate hemocytes from tissues or confirm that they are hemocytes. In the future, when molecular markers of hemocytes such as antibodies or aptamers become available, it will be possible to discriminate and separate hemocytes from tissues and elucidate their function and production pathways.

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Table 1. 106 Trinity-genes, which were hit as immune-related genes contained 27 AMPs-related, 23 clotting-related, 28 proPO-related, and 28 lectin-related by blastx

						Average log2-transformed TMM-TPM value of three samples						
		Result of blastx						Gills	Heart		Lymphoid organs	
Gene classification	Trinity-gene	Top hit of homology genes, gene name [spicies]	Identity (%)	E-Value	Bit-Score	Circulating hemocytes	Sessile hemocytes	Tissue	Sessile hemocytes	Tissue	Sessile hemocytes	Tissue
AMPs	DN17517_c0_g1	ABM63362.1 crustin [Litopenaeus schmitti]	36.36	2.60	25.40	9.90	9.71	9.89	9.90	10.24	9.94	10.64
AMPs	DN22532_c0_g1	AME17866.1 CruI-1 [Marsupenaeus japonicus]	41.12	0.00	80.50	8.13	6.29	4.15	3.18	3.43	1.07	-0.02
AMPs	DN23014_c0_g1	BAB85575.1 cyclic AMP-regulated protein like protein, partial [Marsupenaeus japonicus]	56.52	2.60	25.00	-	6.30	8.02	-	-	-	-
AMPs	DN23122_c0_g2	ACP40176.1 crustin Pm5 [Penaeus monodon]	54.55	0.04	30.00	9.00	6.99	5.01	5.59	4.46	3.85	3.34
AMPs	DN24438_c0_g2	ARB15844.1 crustin A [Litopenaeus vannamei]	86.76	0.00	119.00	-	7.09	8.89		-0.99	-0.86	-
AMPs	DN24879_c0_g1	AKE50479.1 AMP-activated protein kinase subunit alpha [Litopenaeus vannamei]	31.49	0.00	87.80	8.29	8.86	6.46	8.08	7.03	7.50	7.06
AMPs	DN25545_c0_g2	ABO93323.1 crustin [Farfantepenaeus subtilis]	44.44	0.00	35.40	-	8.10	9.27	-1.07	2.83	-	-
AMPs	DN26007_c1_g2	BAB85575.1 cyclic AMP-regulated protein like protein, partial [Marsupenaeus japonicus]	97.39	0.00	303.00	9.19	7.56	5.03	6.38	5.94	5.79	6.31
AMPs	DN26212_c0_g1	ANA91277.1 crustinI-5 [Marsupenaeus japonicus]	34.41	0.00	37.70	-0.36	8.92	-	-	-	-	0.53
AMPs	DN26479_c0_g1	ABW88999.1 double WAP domain-containing protein [Marsupenaeus japonicus]	100.00	0.00	186.00	11.74	9.90	7.58	7.20	6.11	6.32	6.72
AMPs	DN26641_c0_g3	AAQ62565.1 antimicrobial peptide penaeidin 2 [Litopenaeus stylirostris]	37.50	0.23	28.10	8.85	7.20	4.59	6.57	5.09	4.97	4.56
AMPs	DN26691_c0_g2	ANA91277.1 crustinI-5 [Marsupenaeus japonicus]	34.15	0.30	25.80	-	9.72	2.08	3.13	-	-	-
AMPs	DN27042_c3_g1	AMH87234.1 penaeidin-II [Marsupenaeus japonicus]	100.00	0.00	116.00	14.90	12.18	10.33	10.05	9.33	8.96	9.00
AMPs	DN27203_c0_g1	ASR74830.1 antilipopolysaccharide factor E1 [Marsupenaeus japonicus]	84.78	0.00	82.80	2.82	3.68	2.72	2.97	2.73	6.55	8.02
AMPs	DN27203_c0_g3	ANA91279.1 ALF-C1 [Marsupenaeus japonicus]	100.00	0.00	207.00	9.87	8.58	6.26	8.39	6.72	8.74	10.04
AMPs	DN27378_c2_g3	ASR74831.1 antilipopolysaccharide factor E2 [Marsupenaeus japonicus]	38.64	0.45	27.30	9.10	9.06	6.54	9.18	8.09	7.44	8.29
AMPs	DN27452_c0_g1	AOF79108.1 anti lipopolysaccharide factor [Marsupenaeus japonicus]	100.00	0.00	174.00	8.96	8.89	9.53	8.39	8.46	9.09	10.03
AMPs	DN27716_c2_g1	ACY64754.1 double WAP domain-containing protein [Fenneropenaeus chinensis]	39.02	0.24	30.40	9.44	8.08	8.08	7.93	7.97	7.70	8.66
AMPs	DN27929_c0_g1	ABC33920.1 penaeidin 3-2 [Fenneropenaeus chinensis]	41.38	4.40	23.50	9.00	8.74	6.23	8.56	6.64	8.40	7.51
AMPs	DN28400_c0_g3	ABW88999.1 double WAP domain-containing protein [Marsupenaeus japonicus]	60.98	0.00	55.10	5.71	10.55	6.08	2.36	0.47	1.76	
AMPs	DN29063_c0_g1	BAD15063.1 crustin-like peptide type 2 [Marsupenaeus japonicus]	100.00	0.00	197.00	11.84	9.48	7.08	6.89	6.29	6.04	5.67
AMPs	DN29121_c9_g1	ACY64754.1 double WAP domain-containing protein [Fenneropenaeus chinensis]	35.14	1.10	26.90	-	7.52	8.80	-	-	-	-

Table 1. Continued

						Average log2-transformed TMM-TPM value of three samples						
		Result of blastx					Gills		Heart		Lymphoid organs	
Gene classification	Trinity-gene	Top hit of homology genes, gene name [spicies]	Identity (%)	E-Value	Bit-Score	Circulating hemocytes	Sessile hemocytes	Tissue	Sessile hemocytes	Tissue	Sessile hemocytes	Tissue
AMPs	DN29547_c0_g1	AAP33450.1 penaeidin 3-1 [Fenneropenaeus chinensis]	41.67	0.35	28.50	6.52	7.81	6.92	8.59	8.37	9.77	9.82
AMPs	DN30530_c3_g1	AKE50481.1 AMP-activated protein kinase subunit gamma [Litopenaeus vannamei]	98.42	0.00	1004.00	5.24	7.54	8.47	6.37	6.65	6.32	6.48
AMPs	DN30744_c0_g1	BAC57467.1 c-type lysozyme [Marsupenaeus japonicus]	100.00	0.00	270.00	7.46	9.77	7.77	11.45	9.91	9.54	9.49
AMPs	DN31585_c0_g1	AKE50481.1 AMP-activated protein kinase subunit gamma [Litopenaeus vannamei]	26.72	3.30	28.90	8.11	6.13	3.21	3.55	2.66	4.28	4.79
AMPs	DN31816_c2_g1	ANA91278.1 ALF-A1 [Marsupenaeus japonicus]	100.00	0.00	130.00	5.68	8.38	9.51	8.47	8.33	9.27	10.35
Clotting	DN24230_c0_g1	ABK59925.1 clottable protein [Marsupenaeus japonicus]	21.16	1.00	29.30	1.72	8.38	7.78	-	-	-	-
Clotting	DN25562_c0_g1	BAD36808.1 transglutaminase, partial [Marsupenaeus japonicus]	34.38	9.30	23.10	0.69	8.12	9.27	-	2.56	-	-
Clotting	DN25724_c4_g1	BAC99073.1 alpha2-macroglobulin homolog [Marsupenaeus japonicus]	99.57	0.00	425.00	11.26	9.96	6.29	7.18	5.27	6.41	4.84
Clotting	DN25724_c4_g2	BAC99073.1 alpha2-macroglobulin homolog [Marsupenaeus japonicus]	89.33	0.00	137.00	10.45	8.91	4.87	6.43	3.62	5.59	3.67
Clotting	DN27055_c2_g1	BAD36808.1 transglutaminase, partial [Marsupenaeus japonicus]	100.00	0.00	257.00	10.11	8.00	4.79	5.10	4.92	4.64	4.43
Clotting	DN27220_c7_g1	BAD36808.1 transglutaminase, partial [Marsupenaeus japonicus]	43.24	0.64	29.30	-	6.80	8.09	0.92	4.87	5.94	5.43
Clotting	DN27342_c5_g1	ACU31810.1 alpha2 macroglobulin isoform 2 [Fenneropenaeus chinensis]	37.84	0.10	32.30	8.98	7.07	5.77	6.51	5.64	6.15	6.31
Clotting	DN28152_c0_g1	ABW77320.1 clottable protein 2 [Penaeus monodon]	44.44	0.41	29.30	3.72	4.35	5.91	7.24	8.97	2.97	4.32
Clotting	DN29107_c1_g1	ABD92928.1 hemocyte transglutaminase [Marsupenaeus japonicus]	99.44	0.00	1463.00	10.51	8.76	5.53	5.54	5.70	6.56	6.45
Clotting	DN29341_c0_g1	ADT91769.1 alpha 2-macroglobulin [Fenneropenaeus indicus]	35.56	4.20	22.70	-	-	3.09	8.58	9.51	-	3.22
Clotting	DN29516_c4_g1	sp|Q9U572.1|CLOT PENMO RecName: Full=Hemolymph clottable protein; Flags: Precursor	35.48	4.60	25.80	8.46	7.94	8.53	7.74	8.40	8.23	8.85
Clotting	DN30674_c0_g2	ABN13875.1 hemocyte transglutaminase [Litopenaeus vannamei]	50.12	0.00	408.00	0.09	6.52	7.44	6.25	6.83	8.65	8.74
Clotting	DN30674_c0_g3	ACZ71260.1 hemocyte transglutaminase, partial [Fenneropenaeus indicus]	33.77	0.00	58.90	-	6.36	7.47	6.71	6.65	8.53	8.47
Clotting	DN30805_c4_g1	ABC86572.1 alpha 2 macroglobulin, partial [Penaeus monodon]	28.57	2.40	27.30	2.11	4.30	5.19	6.81	8.02	4.86	5.15
Clotting	DN31349_c1_g1	ACU31810.1 alpha2 macroglobulin isoform 2 [Fenneropenaeus chinensis]	91.26	0.00	1420.00	-0.17	4.71	5.51	8.36	8.69	7.05	6.78
Clotting	DN31388_c0_g1	ABK59925.1 clottable protein [Marsupenaeus japonicus]	99.46	0.00	3321.00	1.01	8.77	9.18	9.03	9.18	7.89	8.37
Clotting	DN31512_c7_g1	BAC99073.1 alpha2-macroglobulin homolog [Marsupenaeus japonicus]	98.39	0.00	868.00	12.01	10.52	7.02	7.44	5.90	6.77	5.66
Clotting	DN31512_c7_g2	BAC99073.1 alpha2-macroglobulin homolog [Marsupenaeus japonicus]	99.48	0.00	751.00	12.12	10.50	7.09	7.75	6.04	6.97	5.76
Clotting	DN31515_c0_g3	ABI95361.1 hemolymph clottable protein [Litopenaeus vannamei]	26.00	6.80	24.60	-	5.63	5.52	3.24	4.31	9.39	8.66
Clotting	DN31633_c3_g2	BAC99073.1 alpha2-macroglobulin homolog [Marsupenaeus japonicus]	98.70	0.00	848.00	11.62	10.43	6.91	7.84	6.05	6.99	5.61

Table 5. Continued

						Average log2-transformed TMM-TPM value of three samples						
		Result of blastx					Gills		Heart		Lymphoid organs	
Gene classification	Trinity-gene	Top hit of homology genes, gene name [spicies]	Identity (%)	E-Value	Bit-Score	Circulating hemocytes	Sessile hemocytes	Tissue	Sessile hemocytes	Tissue	Sessile hemocytes	Tissue
Clotting	DN31966_c1_g1	BAD36808.1 transglutaminase, partial [Marsupenaeus japonicus]	99.64	0.00	516.00	12.28	10.17	7.48	7.69	6.79	7.26	6.65
Clotting	DN31966_c2_g1	BAD36808.1 transglutaminase, partial [Marsupenaeus japonicus]	97.43	0.00	556.00	12.51	10.55	7.35	7.94	6.96	7.44	6.90
Clotting	DN32074_c1_g1	ACU31810.1 alpha2 macroglobulin isoform 2 [Fenneropenaeus chinensis]	35.97	0.00	847.00	-4.64	8.20	7.49	-2.35	-3.84	-2.68	-
proPO	DN23662_c0_g1	AGI42860.1 prophenoloxidase 3 [Fenneropenaeus chinensis]	45.16	0.24	28.50	7.12	6.86	7.51	8.34	9.89	6.46	7.38
proPO	DN25445_c0_g1	AAM77689.1 prophenoloxidase [Penaeus monodon]	48.15	0.90	28.50	-1.00	10.02	10.53	-0.14	2.56	-	-0.71
proPO	DN25529_c0_g2	ACL00586.1 prophenoloxidase 2 [Penaeus monodon]	26.67	0.97	29.30	6.16	6.02	5.96	8.02	8.64	5.63	6.00
proPO	DN25967_c0_g2	AAD45201.1 prophenoloxidase [Penaeus monodon]	37.50	6.40	24.30	-	8.07	8.52	-	-	-	-
proPO	DN26279_c3_g7	BAO96441.1 prophenoloxidase beta [Penaeus monodon]	37.21	0.23	26.60	-	7.84	9.75	-	-	2.30	2.29
proPO	DN27763_c6_g1	AFW98993.1 prophenoloxidase activating factor [Litopenaeus vannamei]	43.52	0.00	287.00	8.33	7.34	6.49	5.16	4.62	3.63	1.80
proPO	DN27767_c0_g1	AIU96362.1 peroxinectin [Fenneropenaeus merguiensis]	37.82	0.00	385.00	-1.65	7.56	8.12	-2.58	-	-2.76	-1.12
proPO	DN27947_c4_g3	AAD45201.1 prophenoloxidase [Penaeus monodon]	50.00	7.30	27.30	1.13	4.37	2.59	10.40	12.62	1.46	2.88
proPO	DN28097_c2_g1	AGN53342.1 peroxinectin [Fenneropenaeus indicus]	25.84	1.50	28.90	0.36	7.46	9.09	-1.93	-	-0.58	-0.61
proPO	DN28234_c2_g1	ADR74382.1 prophenoloxidase-activating enzyme 2a [Penaeus monodon]	34.09	0.06	32.30	-2.23	8.52	6.06	-0.21	-1.39	-	-0.75
proPO	DN28260_c0_g3	ACP19559.1 prophenoloxidase-activating enzyme 2 [Penaeus monodon]	34.16	0.00	140.00	-	8.08	7.66	-0.82	-	-0.27	-0.96
proPO	DN28681_c5_g1	AIU96362.1 peroxinectin [Fenneropenaeus merguiensis]	35.71	7.10	27.70	9.57	8.31	5.59	7.57	5.94	7.92	8.15
proPO	DN28723_c0_g1	ABE03741.1 prophenoloxidase activating factor [Penaeus monodon]	89.72	0.00	679.00	10.11	9.62	9.36	7.06	5.26	9.21	9.00
proPO	DN28723_c0_g2	AFH40332.2 prophenoloxidase activating factor, partial [Litopenaeus vannamei]	77.14	0.00	71.20	9.57	8.89	8.66	6.16	5.25	8.73	8.62
proPO	DN29260_c0_g2	AAL05973.1 peroxinectin [Penaeus monodon]	36.70	0.00	374.00	-1.19	7.14	9.08	3.95	1.42	-0.99	-2.52
proPO	DN29474_c4_g3	AFW98992.1 prophenoloxidase activating enzyme 2 [Litopenaeus vannamei]	28.38	5.90	26.20	7.05	7.24	6.62	7.81	6.55	8.43	9.12
proPO	DN30206_c1_g3	ADR74381.1 prophenoloxidase-activating enzyme 1a [Penaeus monodon]	35.93	0.00	128.00	0.71	10.54	9.99	-	1.62	0.06	-0.74
proPO	DN30298_c2_g1	AFW98991.1 prophenoloxidase activating enzyme [Litopenaeus vannamei]	88.91	0.00	682.00	8.94	8.77	9.37	5.12	4.03	4.46	4.62
proPO	DN30664_c0_g1	BAO96441.1 prophenoloxidase beta [Penaeus monodon]	50.00	4.90	23.50	6.92	10.30	4.99	7.12	4.81	6.08	4.22
proPO	DN30664_c0_g4	BAO96441.1 prophenoloxidase beta [Penaeus monodon]	45.83	0.98	24.60	8.89	11.66	7.29	9.14	5.81	7.60	6.75
proPO	DN31165_c0_g3	ACP19558.1 prophenoloxidase-activating enzyme [Penaeus monodon]	45.45	0.01	32.70	-	8.23	8.38	-	-	-	-
proPO	DN31165_c0_g4	AFW98991.1 prophenoloxidase activating enzyme [Litopenaeus vannamei]	43.93	0.00	186.00	-2.43	8.59	8.84	1.23	-	-0.55	-0.69

Table 5. Continued

						Average log2-transformed TMM-TPM value of three samples						
		Result of blastx					Gills		Heart		Lymphoid organs	
Gene classification	Trinity-gene	Top hit of homology genes, gene name [spicies]	Identity (%)	E-Value	Bit-Score	Circulating hemocytes	Sessile hemocytes	Tissue	Sessile hemocytes	Tissue	Sessile hemocytes	Tissue
proPO	DN31298_c1_g2	BAB70485.1 prophenoloxidase [Marsupenaeus japonicus]	100.00	0.00	665.00	11.80	9.65	6.57	7.07	6.10	6.33	5.92
proPO	DN31298_c1_g3	BAB83773.1 prophenoloxidase [Marsupenaeus japonicus]	100.00	0.00	147.00	11.37	9.26	6.23	6.93	5.56	6.47	5.39
proPO	DN31441_c0_g3	ABY81277.1 prophenoloxidase-2 [Litopenaeus vannamei]	87.70	0.00	798.00	11.93	9.98	7.49	7.33	6.31	6.66	6.22
proPO	DN31474_c0_g2	ACM61983.1 prophenoloxidase [Fenneropenaeus chinensis]	39.39	8.70	26.20	11.31	9.35	7.37	9.87	9.70	8.25	8.08
proPO	DN31522_c0_g1	AAM77689.1 prophenoloxidase [Penaeus monodon]	48.39	2.80	27.30	8.60	6.69	4.57	4.37	3.37	3.27	2.37
proPO	DN32004_c1_g1	ACP19558.1 prophenoloxidase-activating enzyme [Penaeus monodon]	41.98	0.00	179.00	6.85	8.37	7.31	5.19	3.26	5.97	5.47
Lectin	DN22119_c0_g1	AHA83582.1 C-type lectin [Marsupenaeus japonicus]	58.21	0.00	155.00	-1.80	7.41	8.23	-	-	-	-0.60
Lectin	DN23268_c0_g1	ADW08727.1 C-type lectin-2 [Litopenaeus vannamei]	30.30	1.20	26.90	-	5.20	8.30	-	-	-	-
Lectin	DN24312_c0_g1	AHA85979.1 C-type lectin 4 [Marsupenaeus japonicus]	99.60	0.00	467.00	-	8.50	9.78	-0.08	0.15	-0.51	-0.77
Lectin	DN24403_c0_g1	AAZ29608.1 C-type lectin [Penaeus monodon]	33.68	0.00	87.80	-2.02	6.69	7.79	6.62	8.69	3.50	3.02
Lectin	DN25052_c0_g1	AAX63905.1 C-type lectin protein [Fenneropenaeus chinensis]	31.94	0.29	32.00	5.42	6.22	6.39	6.45	6.33	8.73	8.51
Lectin	DN25466_c0_g1	AFJ59946.1 C-type lectin 2 [Marsupenaeus japonicus]	38.89	0.00	43.10	-	0.33	0.95	7.73	8.79	2.79	3.95
Lectin	DN26812_c0_g1	ABA54612.1 C-type lectin 1 [Fenneropenaeus chinensis]	45.83	0.92	25.80	-	11.40	6.10	-	2.01	-	1.10
Lectin	DN27060_c0_g1	ANE31673.1 C-type lectin [Fenneropenaeus merguiensis]	36.72	0.00	92.00	0.21	5.22	6.34	11.72	12.79	1.15	1.40
Lectin	DN28092_c0_g1	AGS42195.1 C-type lectin 4 [Fenneropenaeus merguiensis]	29.38	0.00	75.90	-1.07	1.73	2.59	9.35	9.68	6.13	5.90
Lectin	DN28164_c0_g1	ANE31673.1 C-type lectin [Fenneropenaeus merguiensis]	77.22	0.00	379.00	10.19	9.00	6.07	11.06	11.50	5.93	4.76
Lectin	DN28477_c0_g1	AHA83583.1 C-type lectin 2 [Marsupenaeus japonicus]	98.98	0.00	612.00	4.03	4.57	4.23	7.96	8.53	6.79	6.47
Lectin	DN28516_c2_g1	AAZ29608.1 C-type lectin [Penaeus monodon]	40.54	0.00	114.00	-1.86	9.78	11.08	6.90	8.15	7.34	7.77
Lectin	DN28517_c0_g1	AFJ59947.1 C-type lectin 3, partial [Marsupenaeus japonicus]	99.72	0.00	752.00	-	3.84	4.59	6.37	6.86	8.92	9.43
Lectin	DN29044_c3_g1	AFJ59946.1 C-type lectin 2 [Marsupenaeus japonicus]	100.00	0.00	146.00	6.26	7.16	6.01	9.38	9.39	9.71	10.09
Lectin	DN29044_c3_g2	AFJ59946.1 C-type lectin 2 [Marsupenaeus japonicus]	100.00	0.00	134.00	6.93	8.40	6.32	9.73	8.50	10.56	11.09
Lectin	DN29306_c1_g1	BAW18769.1 gill C-type lectin [Marsupenaeus japonicus]	39.82	0.00	253.00	4.60	4.83	3.17	10.53	10.95	8.62	9.14
Lectin	DN29551_c0_g3	AFJ59946.1 C-type lectin 2 [Marsupenaeus japonicus]	31.71	0.01	34.30	9.18	7.65	5.12	5.52	5.13	5.39	4.61
Lectin	DN29665_c2_g2	ACJ06431.1 C-type lectin 3 [Fenneropenaeus chinensis]	27.72	0.00	48.10	6.64	8.52	6.16	9.81	8.31	10.82	11.65

Table 5. Continued

						Average log2-transformed TMM-TPM value of three samples						
		Result of blastx					Gills		Heart		Lymphoid organs	
Gene classification	Trinity-gene	Top hit of homology genes, gene name [spicies]	Identity (%)	E-Value	Bit-Score	Circulating hemocytes	Sessile hemocytes	Tissue	Sessile hemocytes	Tissue	Sessile hemocytes	Tissue
Lectin	DN29761_c5_g1	AFJ59946.1 C-type lectin 2 [Marsupenaeus japonicus]	39.13	7.00	23.50	9.72	8.27	4.90	7.63	5.83	5.80	5.35
Lectin	DN30279_c0_g2	ASA69502.1 penlectin 5-2 [Penaeus monodon]	41.28	0.00	158.00	-2.88	7.99	9.02	0.03	0.45	-	2.41
Lectin	DN30405_c0_g3	ANE31673.1 C-type lectin [Fenneropenaeus merguiensis]	36.15	0.00	79.30	0.36	0.61	0.22	6.25	7.29	7.83	8.04
Lectin	DN30675_c3_g2	ABV58637.1 C-type lectin-like protein, partial [Metapenaeus ensis]	26.42	6.00	23.90	-0.26	7.97	8.97	-	0.47	0.18	0.04
Lectin	DN31020_c0_g1	AEH05998.1 C type lectin containing domain protein [Litopenaeus vannamei]	44.37	0.00	256.00	8.81	7.95	5.04	7.53	7.78	5.68	5.63
Lectin	DN31125_c0_g1	AAZ29608.1 C-type lectin [Penaeus monodon]	28.07	1.30	29.30	4.34	4.93	5.42	8.48	8.69	4.95	4.67
Lectin	DN31425_c0_g2	ACJ06428.1 C-type lectin 2 [Fenneropenaeus chinensis]	26.92	3.70	24.30	-2.92	3.81	2.16	9.22	0.18	2.41	-
Lectin	DN31539_c0_g1	BAW18769.1 gill C-type lectin [Marsupenaeus japonicus]	99.21	0.00	266.00	0.68	11.76	12.84	3.10	3.40	0.71	1.31
Lectin	DN31539_c0_g3	BAW18769.1 gill C-type lectin [Marsupenaeus japonicus]	90.77	0.00	214.00	-0.08	11.47	12.80	-	1.74	0.23	2.78
Lectin	DN31957_c0_g1	AEH05998.1 C type lectin containing domain protein [Litopenaeus vannamei]	30.19	6.00	27.70	-2.51	8.13	8.78	7.15	7.22	5.55	5.77

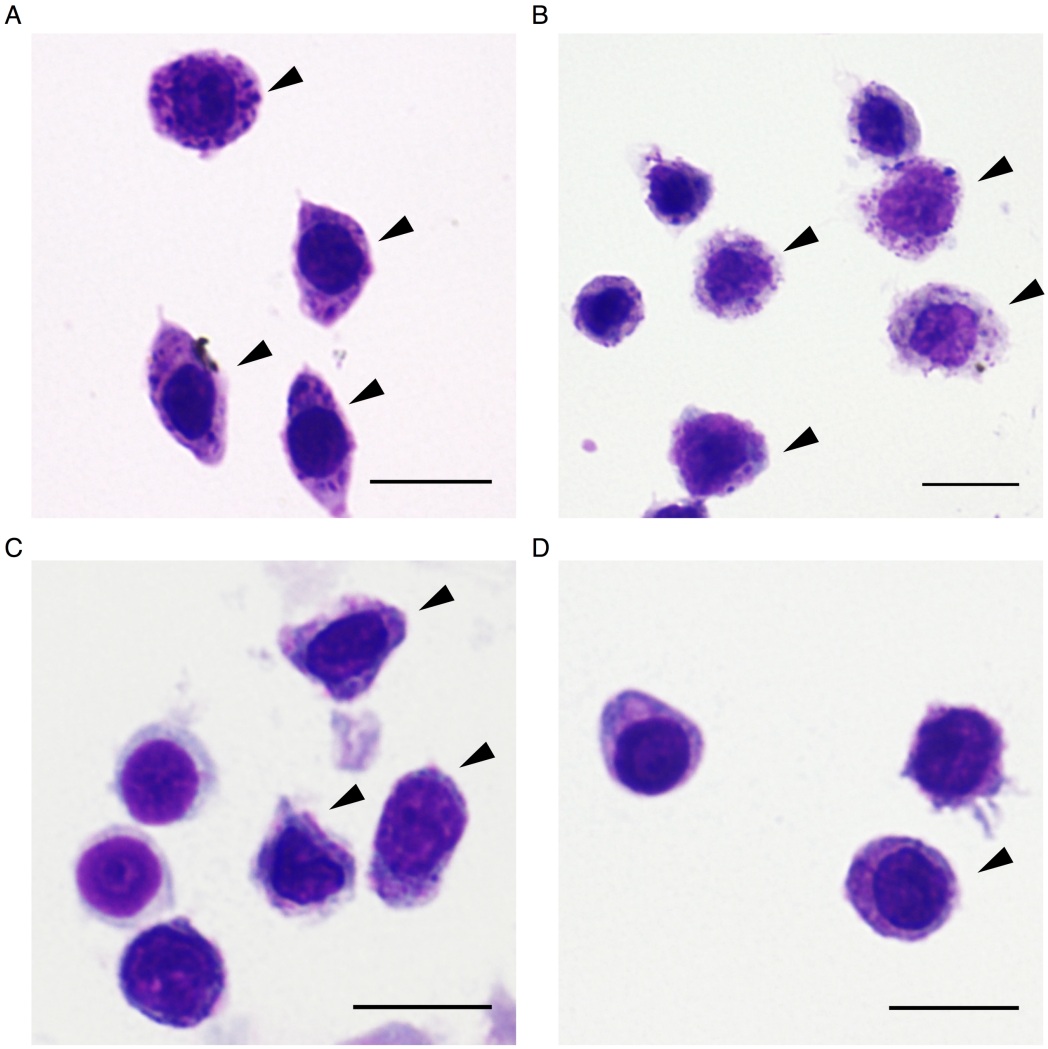

Figure 1. Giemsa staining of circulating and sessile hemocytes of *M. japonicus*. Circulating hemocytes (A), sessile hemocytes of gills (B), heart (C) and lymphoid organs (D) were stained by Giemsa solution. Black arrows indicate hemocytes composed granules in its cytoplasm. Bars indicate 10 μm scale.

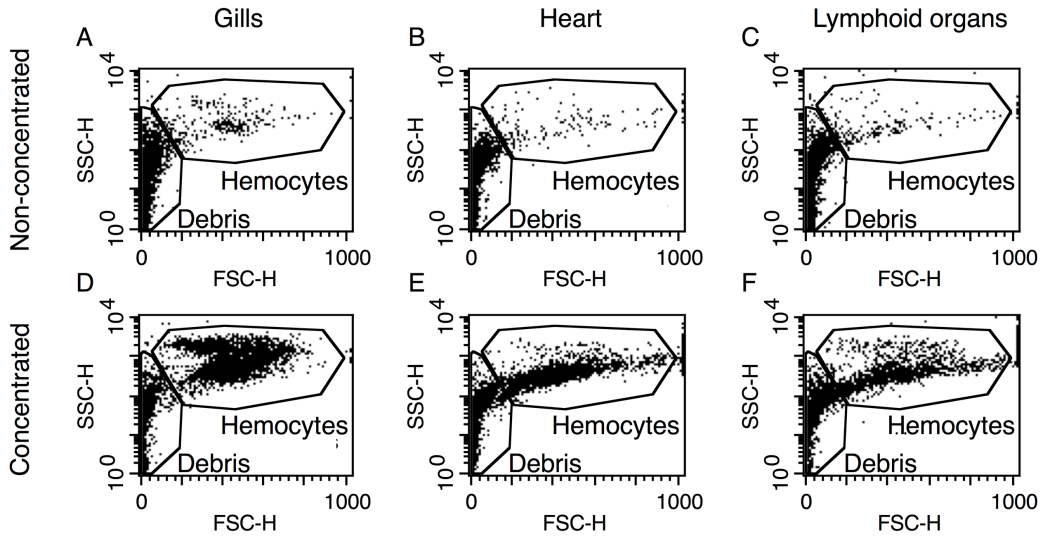

Figure 2. Dot plots analyses of non-concentrated and concentrated sessile hemocytes of gills, heart and lymphoid organs. The target tissue was gills (A, D), heart (B, E) and lymphoid organs (C, F), non-concentrated samples (A, B, C) and concentrated samples (D, E, F). X-axis indicates FSC, Y-axis indicates SSC, respectively.

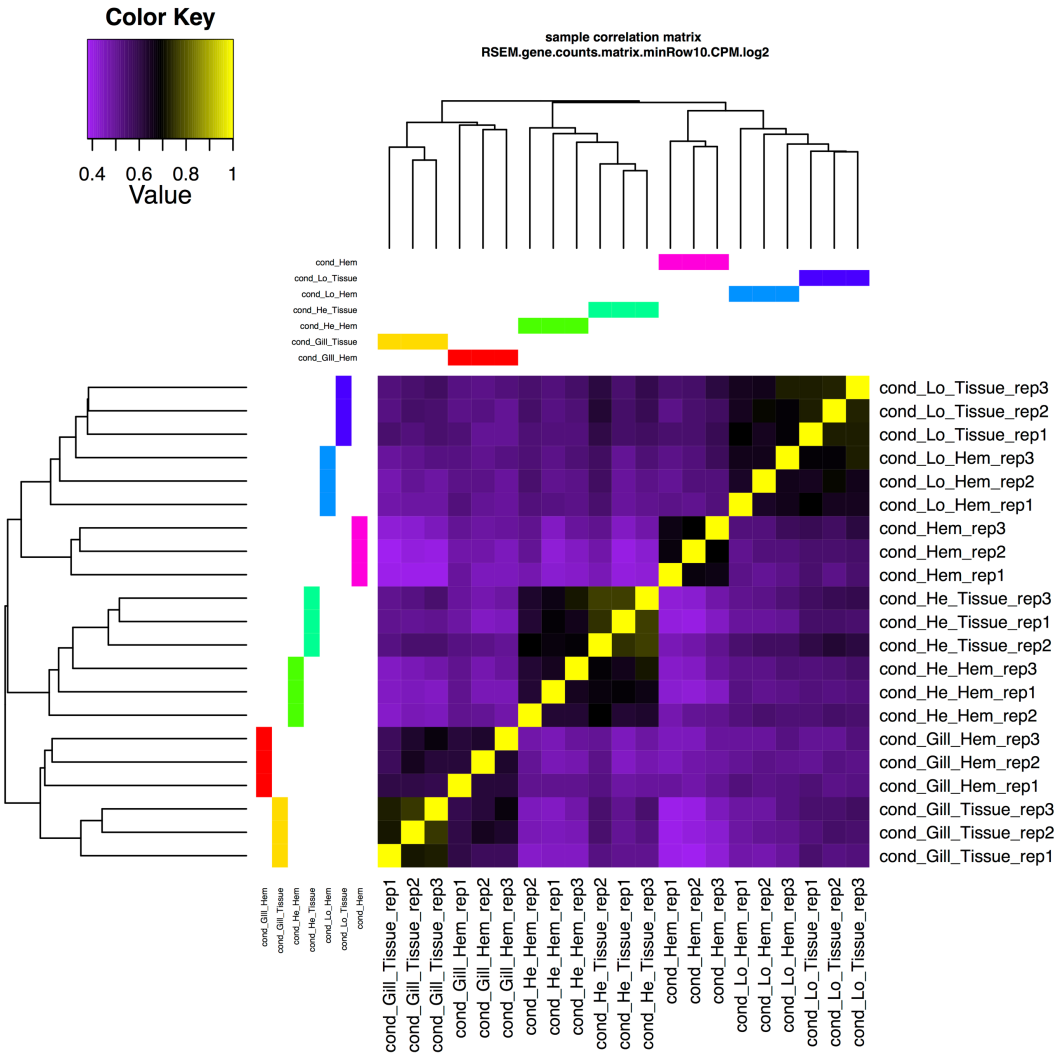

Figure 3. Correlation matrix for all sample replicates. Each condition was clustered together with same conditions.

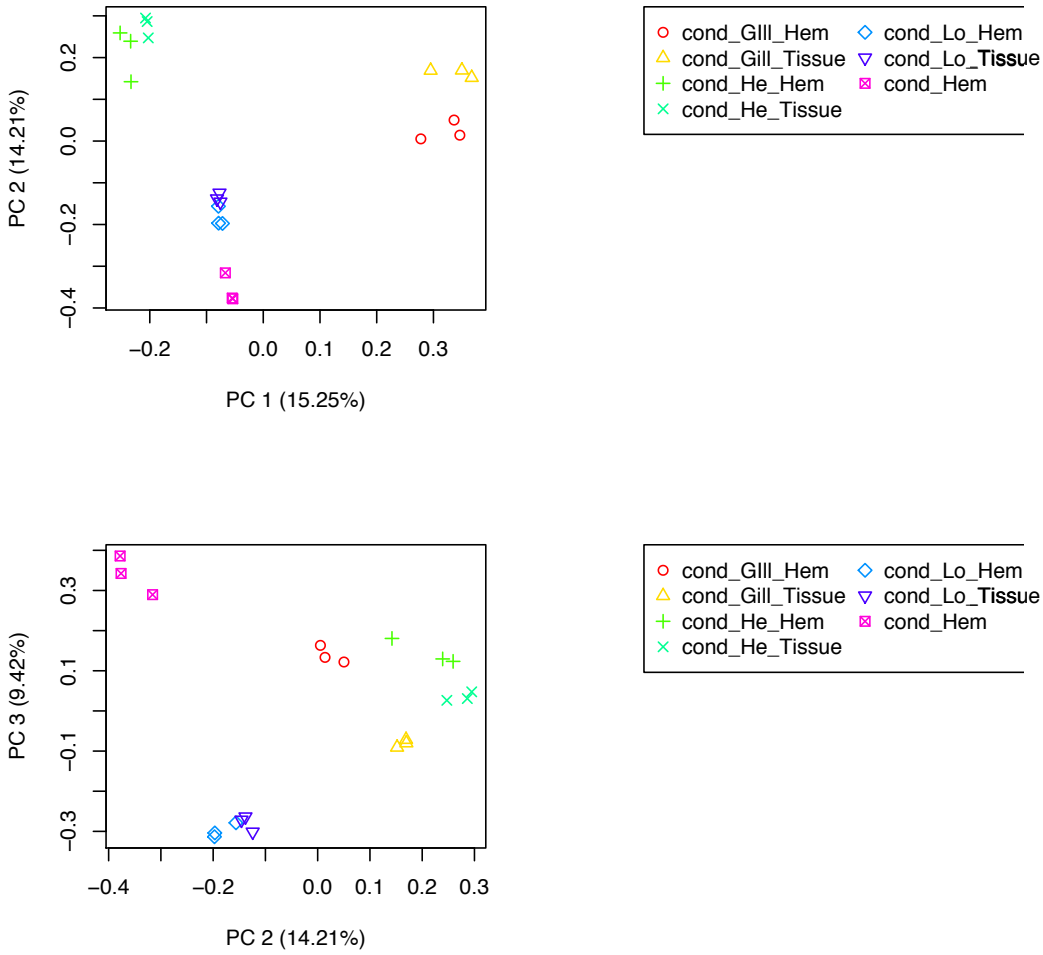

Figure 4. Principal component analysis (PCA) plot result across all samples. Each sample replicate was grouped together with same sample type.

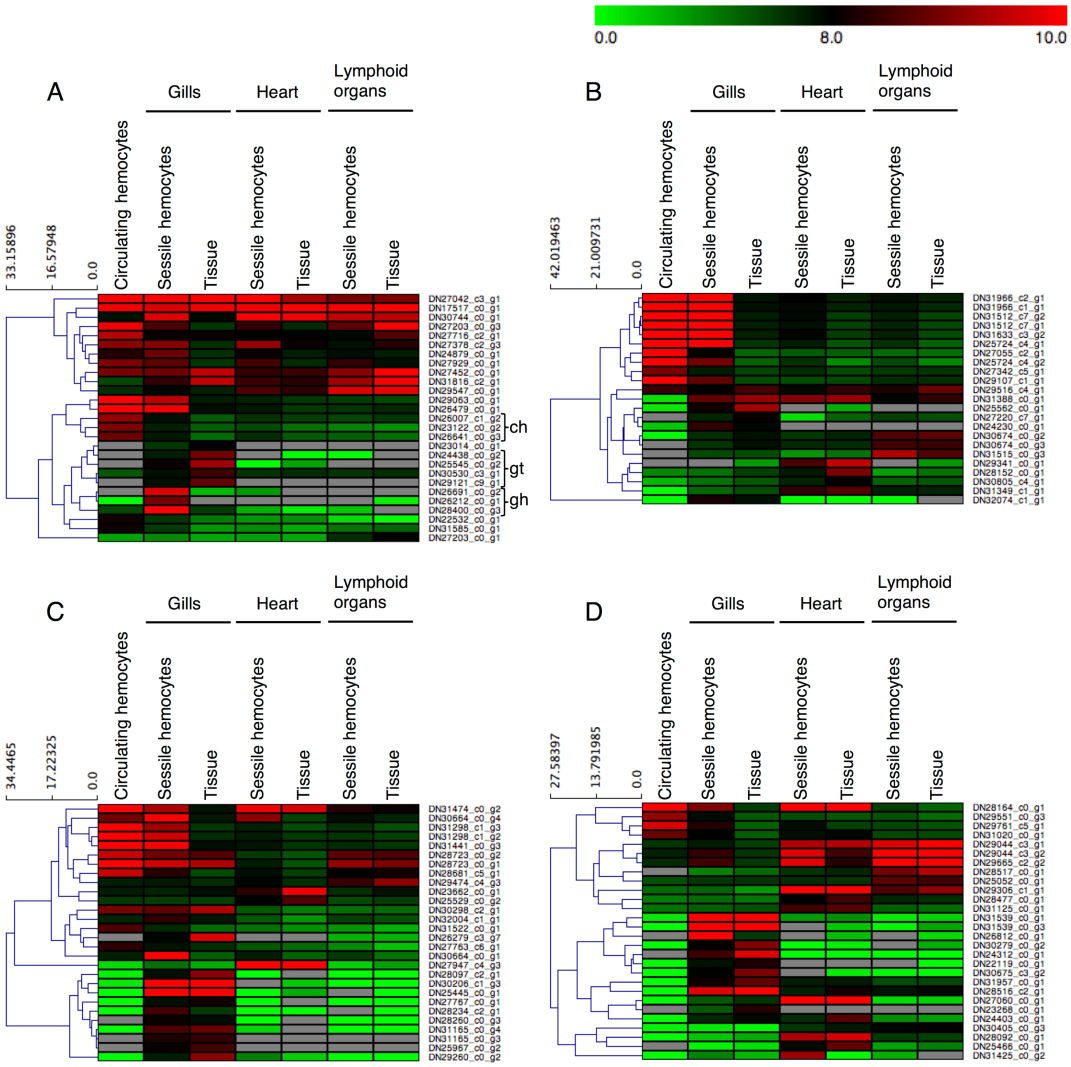

Figure 5. Hierarchical clustering analysis of immune-related Trinity-genes in circulating hemocytes, sessile hemocytes of gills, heart, lymphoid organs and each tissue. AMP-related (A), clotting-related (B), proPO-related (C) and lectin-related (D) Trinity-genes. Each column is the average log2-transformed TMM-TPM (Log TMM-TPM) value of three samples in each experimental group. Relatively highly expressed genes are shown in red, relatively lowly are in green, no-expression are in gray. The symbols indicate Trinity-genes were mainly expressed in ch; circulating hemocytes, gt; tissue of gills, gh; sessile hemocytes of gills, respectively.

第３章　RNA-seq identifies integrin alpha of kuruma shrimp *Marspenaeus japonicus* as a candidate molecular marker for phagocytic hemocytes

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第１節 Abstract

Phagocytosis is main cellular immunity, however, it is still unknown or debated upon which types of hemocyte contributes phagocytosis in penaeid shrimps. The hemocyte characterization in kuruma shrimp have been mainly performed based on its morphology by microscopic observation. Therefore, establishment of molecular markers to distinguish phagocytic hemocytes is required. In this study, using magnetic fluorescent beads, I enriched phagocytic hemocytes and conducted RNA-seq analysis between total and enriched phagocytic hemocytes. The data demonstrated functional difference between total and phagocytic hemocytes. In addition, a transcript homologous to integrin-alpha was highly expressed in phagocytic hemocytes, and named Mj-Intgα. Using anti-serum against Mj-Intgα revealed that around 60% of total hemocytes and more than 90% of phagocytic hemocytes showed positive for Mj-Intgα. This study presents Mj-Intgα as a candidate molecular marker for future functional characterization of hemocytes.

第２節 Introduction

Among crustaceans, shrimp included, circulating hemocytes contribute to both cellular and humoral immune responses, such as phagocytosis, encapsulation, nodule formation, blood coagulation, prophenoloxidase-activating system (proPO) in the melanization cascade, production of antimicrobial peptides (AMPs), opsonins, etc., (for reviews see Jiravanichpaisal *et al.,* 2006; Söderhäll, 2016; Tassanakajon *et al.,* 2013). These circulating hemocytes have been characterized into three types; the hyaline cells (HCs), the semigranular cells (SGCs) and the granular cells (GCs) based on their morphology (Johansson *et al.,* 2000; Söderhäll and Smith, 1983). To study different types of hemocytes, separation techniques of hemocyte have been reported in penaeid shrimp such as Percoll (Bachère *et al.,* 1995; Koiwai *et al.,* 2017; Vargas-Albores *et al.,* 2005) or iodixanol (Dantas-Lima *et al.,* 2013) density gradient centrifugation, cell-sorting by flow cytometry (FCM) (Sun *et al.,* 2010; Yang *et al.,* 2015; Yip and Wang, 2002).

To classify or isolate identical cells, cell surface molecules known as cluster of differentiation (CD) markers are often used especially for mammalian leukocytes (Bock *et al.,* 2012; Laudanski *et al.,* 2006). According to this, monoclonal antibodies (mAbs) have been developed to classify hemocytes types in penaeid shrimp (Rodriguez *et al.,* 1995; Sung and Sun, 2002; Van de Braak *et al.,* 2000; Winotaphan *et al.,* 2005, Xing *et al.,* 2017). Using these mAbs, hemocytes were divided into two types, mAbs positive and negative, to characterize different hemocyte sub-populations. Whereas, these mAbs couldn’t characterize any distinct function of hemocytes.

Each type of hemocyte are believed to have a distinct immune function (Johansson *et al.,*

2000). Among these immune functions, phagocytosis, the digestion of foreign particles including microorganisms, is a conserved cellular immune response in eukaryotes. Phagocytosis is well studied in mammalian phagocytes include many types of white blood cells such as neutrophils, monocytes, macrophages, mast cells, and dendritic cells (for a review see Aderem and Underhill, 1999). However, in crustaceans, it is still unclear or debated upon on whether which types of hemocyte mediate phagocytosis. Moreover, references from various data from different spices shows conflicting results (for reviews see Jiravanichpaisal *et al.,* 2006; Söderhäll, 2016). Since, there is no molecular markers or a technique precise enough to characterize hemocyte types, thus, the identification of the molecular marker for phagocytic hemocytes will be instrumental.

For studying phagocytosis in penaeid shrimp, some of studies used fluorescence micro beads and FCM (Oliver *et al.,* 2011), such as the injection of magnetic micro beads *in vivo*, resulting to the enrichment of phagocytic immune cells facilitating the proteomic analyses conducted in mosquito *Anopheles gambiae* (Simth *et al.,* 2016).

In this study, I enriched phagocytic hemocytes using fluorescent magnetic beads and conducted transcriptome analysis via RNA-seq analysis. Differences in gene expression between total hemocytes and enriched phagocytic hemocytes were analyzed on the transcriptome profiles to predict possible functions of each hemocytes as well as to identify candidate marker molecules for phagocytic hemocytes. From analysis, one transcript, DN8588_c0_g1 named as Mj-Intgα, was identified as candidate marker of phagocytic hemocytes. Finally, I evaluated the potential of Mj-Intgα as a marker of phagocytic hemocytes through immuno-staining using anti-Mj-Intgα rabbit serum.

第３節 Material and methods

Shrimp samples

Kuruma shrimp (*Marsupenaeus japonicus*) were obtained from farms in Okinawa and Miyazaki prefecture, Japan. Shrimps were kept in tanks provided with water recirculating system maintained at 25 ^{0}C and 30-35 ppt. Shrimps were acclimatized for at least 3 days before the experiment. Apparently healthy shrimp were used in this study.

Enrichment of phagocytic hemocytes by *in vivo* injection of fluorescent magnetic beads

Shrimps (average 25 g) were injected with 100 µL of 10% suspension of fluorescent magnetic beads (FMB) (Fluorescent YG Superparamagnetic Microparticles: Polysciences, Inc., USA) in kuruma shrimp PBS (KPBS) (480 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, pH: 7.4). Three (3) hours post injection, 1 mL of hemolymph was collected from each shrimp using a 23-gauge needle and syringe containing 1 mL of 10 mM EDTA diluted in KPBS (E-KPBS) as anti-coagulant, then diluted the cell concentration to 1 x 10^7 cells/mL by E-KPBS. To enrich the hemocytes engulfed FMB (FMB+ hemocytes), 1 mL of diluted hemocytes was placed on DynaMagTM-2 Magnetic Particle Concentrator (Thermo Fisher Scientific Inc., USA) and incubated 2 min, then discard the supernatant. To wash enriched FMB+ hemocytes, samples were re-suspended in 1 mL of 0.5% albumin from bovine serum (BSA) (Sigma-Aldrich Co., USA) and 10 mM EDTA containing KPBS (B-E-KPBS), and repeated this washing proses twice. After washing three times, hemocytes were then collected as enriched FMB+ hemocytes (EF-Hem) for further studies. Before enrichment, a part of cells (3 x 10^6 cells) were also collected

as total hemocytes (T-Hem). This enrichment was conducted totally ten times, three times for verification of the enrichment process, four times for total RNA collection and three times for immunostaining from ten shrimps individually.

The ratio of FMB+ hemocytes was determined by flow cytometry analysis and fluorescence microscopy (Nikon, Japan) to verify the enrichment process. Both T- and EF-Hem were analyzed for at least 2,500 events by FACSCalibur (Becton-Dickinson, USA) using an FL-1 filter with Cell Quest Pro software ver. 5.2.1 (Becton-Dickinson). The fluorescence of FMB was detected with an FL-1 filter. Then the ratio of FMB+ hemocytes was calculated by Cell Quest Pro software ver. 5.2.1. The cells were examined by bright-field or fluorescent field using upright microscope ELIPSE Ci (Nikon Co., Japan), and the images were analyzed by NIS-Elements (Nikon Co.) and ImageJ ver. 2.0.0. (Schneider *et al.,* 2012). The assay was performed three times from three individual shrimps.

cDNA Library construction and sequencing by Illumina Miseq

T- and EF-Hem from a shrimp were collected as described in above section. Total RNA was extracted using a RNeasy Mini kit (QIAGEN, Germany) following the manufacturer's protocol. The concentration and purity of total RNA were measured using a Qubit® RNA BR Assay Kit (Thermo Fisher Scientific Inc.) and NanoDrop Lite (Thermo Fisher Scientific Inc.). RNA-seq libraries were prepared with 250 ng of total RNA using TruSeq stranded mRNA sample preparation kit (Illumina Inc., USA) followed manufacture's protocol. The libraries were amplified with 15 cycles of PCR and contained indexes within the adaptors. Finally, amplified library yields were measured using a Qubit® dsDNA HS Assay Kit

(Thermo Fisher Scientific Inc.) and D1000 ScreenTape System (Agilent Technologies, USA), had 43.4 ng/μL for T-Hem and 32.2 ng/μL for EF-Hem with an average length of 305 bp for T-Hem and 313 bp for EF-Hem, indicating a concentration of 225.4 nM for T-Hem and 164.2 nM for EF-Hem. The libraries were diluted to 10 nM and sequenced using MiSeq (Illumina Inc.) and MiSeq reagent kit version 2 (Illumina Inc.) with 150 paired end reads.

De novo assembly and identification of differentially expressed transcripts

The reads were assembled by Trinity v2.1.1 (Grabherr *et al.,* 2011) using default parameters (minimum assembled transcripts length 200) to obtain Trinity-assembled transcripts. The sequenced libraries were mapped back to the reference Trinity-assembled transcripts using RSEM (Li and Dewey, 2011) to quantify the read counts. Read counts were TMM-normalized (trimmed mean of M-values) to account for differences in library size (Robinson and Oshlack, 2010) and then TPM-normalized (transcripts per kilobase million) to account for differences in transcript length. The differentially expressed transcripts between T- and EF-Hem library were identified using EdgeR (Robinson *et al.,* 2010). Default parameters were used, including a p-value cutoff for false discovery rate (FDR) of 0.01 and a minimum 2-fold change in expression. Blastx program (Altschul *et al.,* 1997) was then used for homologous gene searching in Penaeidae's 4,438 proteins (downloaded at Feb. 8, 2017) in NCBI database (http://www.ncbi.nlm.nih.gov) and DDBJ (http://www.ddbj.nig.ac.jp/index-j.html).

Validation of RNA-seq results by quantitative RT-PCR (qRT-PCR)

Total RNA of T- and EF-Hem were extracted from three shrimps as described in above section. cDNAs were synthesized from 100 ng of RNA of each sample using a High capacity cDNA reverse transcription kit (Thermo Fisher Scientific Inc.). After synthesis, cDNA samples were diluted 5 times by distilled water and 2 µl of samples were used for qRT-PCR. The set of primers were designed from three transcripts (DN8588_c0_g1, DN9598_c0_g1 and DN10184_c0_g1) and elongation factor 1α (EF-1α: as an internal control) for qRT-PCR (Table 1). Quantitative reverse transcription PCR (qRT-PCR) was conducted using THUNDERBIRD SYBR qPCR Mix (TOYOBO Co. Ltd., Japan) and condition was 95 ^{0}C for 1 min, 40 cycles of 95 ^{0}C for 15 secs and 60 ^{0}C for 1 min followed by dissociation analysis step. mRNA accumulation of each gene was calculated as ΔCT by comparing with CT value of EF-1α (as a reference gene). The statistical significance between T- and EF-Hem was analyzed using t-test.

Molecular cloning, structural analysis and tissue distribution analysis of Mj-Intgα

The open reading frame containing region of DN8588_c0_g1 (named as Mj-Intgα) was cloned with the primer sets (Table 1) designed based on Trinity-assembled transcripts data from above section After sequencing by ABI Genetic Analyzer 3130 (Applied Biosystems Inc., USA), the signal peptide was predicted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/), the protein conserved domain was predicted by Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/), the transmembrane helices were predicted by TMHMM Server v. 2.0.

(http://www.cbs.dtu.dk/services/TMHMM/) and the molecular weight was predicted by ProtParam tool (http://web.expasy.org/protparam/).

The mRNA expression levels of the Mj-Intgα gene was determined by RT-PCR and qRT-PCR. cDNA samples of 9 tissues gills (G), heart (He), hemocytes (H), hepatopancreas (Hp), intestine (I), lymphoid organ (Lo), muscle (M), nerve (N) and stomach (S) were prepared using the same protocol as above section. RT-PCR was conducted using 2 µL of cDNA from various tissues using Mj-Intgα and EF-1α (as an internal control) detection primers (Table 1). The amplification process was conducted under the following PCR conditions: pre-denatured at 95 ^{0}C for 5 min, then amplified for 28 cycles at 95 ^{0}C for 30 secs, 55 ^{0}C for 30 secs, 72 ^{0}C for 30 secs and a final extension at 72 ^{0}C for 5 min. RT-PCR products were viewed by electrophoresis on 1.0% agarose gel. qRT-PCR was also conducted using 1 µL of cDNA from various tissues with Mj-Intgα and EF-1α (as an internal control) primers for qRT-PCR (Table 1) using THUNDERBIRD SYBR qPCR Mix (TOYOBO Co. Ltd., Japan), and condition was 95 ^{0}C for 1 min, 40 cycles of 95 ^{0}C for 15 secs and 60 ^{0}C for 1 min followed by dissociation analysis step. mRNA accumulations of Mj-Intgα on each tissue were calculated as ΔCT by comparing with CT value of EF-1α (as a reference gene), then relative mRNA expressions of Mj-Intgα between tissues were calculated as ΔΔCT by comparing ΔCT value of Mj-Intgα with ΔCT value of Mj-Intgα on muscle (as a reference tissue).

Expression of recombinant Mj-Intgα protein and antiserum preparation

Part of the Mj-Intgα sequence was amplified with the specific primers Mj-Intgα_F_NdeI

and Mj-Intgα_R_NotI from the hemocyte cDNA template. After purification of the target fragments from agarose gel with Gen Elute Agarose Spin Columns (Sigma-Aldrich Co.), the fragments were digested by restriction enzymes NdeI and NotI, then the digested fragment was cloned into the pET-32a vector with adding a His-tag which was digested with the same restriction enzymes. After confirmation of the sequence by ABI Genetic Analyzer 3130, the recombinant plasmid pET-32a-Mj-Intgα was transformed into the *Escherichia coli* BL21 (DE3) strain. The *E. coli* transformed with pET-32a-Mj-Intgα was incubated at 37 ^{0}C overnight and treated with 1 mM of IPTG for an additional 4 h at 37 ^{0}C. The cells were collected, re-suspended in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, pH: 7.4), sonicated for 10 min on ice and centrifuged at 9,000 rpm for 20 min at 4 ^{0}C. The pellet was washed 3 times by PBS and dissolved in denaturation buffer (8 M Urea, 50 mM Tris-HCl, 0.1% SDS, pH: 8.2). From the dissolved protein, recombinant Mj-Intgα (rMj-Intgα) was purified by Ni-NTA agarose (QIAGEN) followed manufacture's protocol. The purity of rMj-Intgα was checked by running it on a 10% SDS-PAGE gel and its concentration was measured by Qubit® Protein Assay Kit (Thermo Fisher Scientific Inc., USA). Anti-rMj-Intgα serum was developed by immunizing a rabbit with rMj-Intgα (Eurofins genomics Co. Ltd., Japan). To check reactivity of anti-rMj-Intgα serum against rMj-Intgα, 200 ng of rMj-Intgα was analyzed by 10% SDS-PAGE gel, and transferred to a polyvinylidene difluoride membrane (ATTO Co., Japan). The membrane was blocked with blocking buffer containing 5% BSA in TBS-T (137 mM NaCl, 2.7 mM KCl, 50 mM Tris-HCl, 0.05% Tween 20, pH: 7.4) for 1 h at room temperature, incubated in the same buffer with anti-rMj-Intgα serum or non-immunized rabbit serum (1:10,000 diluted in TBS-T) for

1 h at room temperature, washed 3 times with TBS-T, incubated in the same buffer with anti-rabbit IgG (Fc) AP conjugate (1:5,000 diluted in TBS-T) (Promega Co.) for 30 min at room temperature, washed 3 times with TBS-T, and stained with SIGMAFAST BCIP/NBT tablet (Sigma-Aldrich Co., USA).

Immunostaining of Mj-Intgα on hemocytes

T- and EF-Hem were prepared according to the protocol as above section. except that the anti-coagulant of E-KPBS to fixing solution of 2% paraformaldehyde and 10 mM EDTA containing KPBS for fixing hemocytes. Both T- and EF-Hem were diluted to the concentration of 1 x 10^6 cells/mL by 0.5% BSA containing KPBS (B-KPBS). An aliquot of each hemocyte suspension (150 μL) was spread on a glass slide using cell collection bucket SC-2 (TOMY SEIKO Co. Ltd., Japan) at 300 rpm for 2 min. Hemocytes on the glass slide were incubated with anti-rMj-Intgα serum (1:100 diluted in B-KPBS) for 1 h at room temperature, washed 3 times with B-KPBS, incubated with goat anti-rabbit IgG H&L (Alexa Fluor® 568) (Abcam plc., UK) (1:500 diluted in B-KPBS) for 30 min at room temperature, washed 3 times with TBS-T, and stained nucleolus with Hoechst 33258 (Thermo Fisher Scientific Inc.). For negative control, no anti-serum was replaced anti-rMj-Intgα serum. Stained glass slide samples were examined by bright-field or fluorescent field using upright microscope ELIPSE Ci, and the pictures were analyzed by NIS-Elements and ImageJ ver. 2.0.0. The ratio of Mj-Intgα positive hemocytes (Mj-Intgα+ hemocytes) and Mj-Intgα negative hemocytes (Mj-Intgα- hemocytes) was count from randomly selected more than 100 hundred hemocytes on both T- and EF-Hem. The assay was performed three times from

three individual shrimps.

第４節 Results

Enrichment of FMB+ hemocytes

The percent of total hemocytes (T-Hem) that were positive for fluorescent magnetic beads (FMB+) was 6.4 ± 2.3% (Fig. 1A). The percent of the magnetically separated hemocytes, called enriched FMB+ hemocytes (EF-Hem), that were FMB+ was 69.6 ± 8.6% (Fig. 1C). The enrichment process was confirmed by fluorescence microscopy, demonstrating the actual phagocytosis of FMB by hemocytes (Fig. 1B, D).

De novo assembly and identification of differentially expressed transcripts

The assembled transcripts contained 44,088 transcripts. The mean transcript length was 411 bp and the N50 (weighted median) was 1,477 bp. From the result of analyzing differentially expressed transcripts, 11 transcripts were identified based on p-value cut-off for FDR of 0.01 and a minimum 2-fold change in expression (Table 2). One (1) transcript showed higher expression in EF-Hem and 10 transcripts showed higher expression in T-Hem. Contig number, log2-transformed fold change (Log FC), log2-transformed counts per million (Log CPM), false discovery rate (FDR) and homology gene which was found in NCBI and DDBJ database are shown in Table 2.

Validation of differentially expressed transcripts by qRT-PCR

In the case of transcript DN8588_c0_g1, which showed higher expression in EF-Hem, showed higher ΔCt value in EF-Hem than T-Hem in qRT-PCR (Fig. 2). On the other hand, transcripts DN9598_c0_g1 and DN10184_c0_g1, which showed higher expression in T-

Hem, showed higher ΔCt value in T-Hem than EF-Hem in qRT-PCR (Fig. 2). In addition, the p-value of the differentiation of 2 transcripts, DN8588_c0_g1 and DN9598_c0_g1, were less than 0.01, and transcript DN10184_c0_g1, was less than 0.05 based on statistical analysis. These results of qRT-PCR confirm the significant difference between differentially expressed transcripts of the RNA-seq analysis.

Molecular characteristics and tissue distribution profile of Mj-Intgα

The amino acid sequence of Mj-Intgα (GenBank No. LC114983) consisted 1,111 amino acids, including one signal peptide (1-19th aa), three integrin alpha domains (34-93th aa, 370-424th aa and 431-486th aa) and one transmembrane domain (1,055-1,077th aa) (Fig. 3A). Predicted molecular weight of Mj-Intgα except signal peptide was about 116 kDa.

The distribution of Mj-Intgα mRNA in 9 tissues in apparently healthy shrimp were examined by RT-PCR and qRT-PCR. The Mj-Intgα mRNA was detected or highly accumulated in heart, hemocytes and lymphoid organs (Fig. 3B, C).

Reactivity of anti-rMj-Intgα serum against rMj-Intgα

Based on the detections of SDS-PAGE analysis, the rMj-Intgα was found to be pure (Fig. 4A lane 1). The result of western blotting analysis showed that the anti-rMj-Intgα serum bound to rMj-Intgα (Fig. 4B lane 1) but non-immunized rabbit serum didn’t bind to rMj-Intgα (Fig. 4B lane 2).

Immunostaining of Mj-Intgα on hemocytes

Anti-rMj-Intgα staining showed that Mj-Intgα was localized on the hemocyte cell surface (Fig. 5). The ratio of Mj-Intgα+ hemocyte was 73.5 ± 8.3% on T-Hem and 93.4 ± 5.4% on EF-Hem, respectively. About 5% on EF-Hem were also Mj-Intgα- FMB+ (Fig. 5D indicated by green arrow).

第５節 Discussion

By using FMB, I was able to enrich and isolate phagocytic hemocytes. Compared to other techniques such as gradient centrifugation or cell-sorting, our method does not need expensive equipment. Therefore, this technique can be used to study phagocytic hemocytes in other invertebrates.

Eleven transcripts were found to be highly expressed in T-Hem, with six of them being homologous to immune-related genes. DN10184_c0_g1 has a role in blood coagulation (Yeh *et al.,* 2006), while the other five (DN9598_c0_g1, DN10529_c1_g8, DN10956_c3_g7, DN8333_c0_g1 and DN5567_c0_g1) have roles in the proPO system (Amparyup *et al.,* 2007; Pang *et al.,* 2014; Rattanachai *et al.,* 2004; Shanthi and Vaseeharan, 2014). In *Litopenaeus vannamei*, granular cells (GCs) were the main producers of proPO transcripts while hyaline cells (HCs) were the main producers of blood coagulation-related transcripts (Yang *et al.,* 2015). Therefore, EF-Hem may consist mainly of semigranular cells (SGCs). It is also possible that phagocytosis decreased gene expression by hemocytes. To test these hypotheses, it is necessary to develop a robust method for classifying HCs, SGCs and GCs based on mAbs and to confirm the expression levels at the protein level.

One transcript was highly expressed in EF-Hem. Because it was homologous to Integrin alpha 5 of *Fenneropenaeus chinensis* (Sun *et al.,* 2014), it was named Mj-Intgα. Integrins are αβ heterodimeric cell surface receptors that mediate cell-cell and cell-matrix interactions and serve essential cell adhesion functions (reviewed in Huhtala *et al.,* 2005; Johnson *et al.,* 2009). In vertebrates, some integrins have been used as cluster of differentiation (CD) markers including some that are specific for leukocytes (reviewed in Barczyk *et al.,* 2010;

Luo *et al.,* 2007). Integrins have been found to mediate phagocytosis in oyster (Jia *et al.,* 2015), crab (Huang *et al.,* 2015), *Drosophila* (Nonaka *et al.,* 2013) and *C. elegans* (Hsu & Wu, 2010). In addition to being expressed in hemocytes, Mj-Intgα transcripts were also expressed in the heart and lymphoid organ (Fig. 3B, C), both of which are known to have phagocytic activity and/or phagocytic hemocytes (Kondo *et al.,* 1998; Supamattaya *et al.,* 2003), in agreement with our finding of Mj-Intgα mRNA expressed in phagocytic hemocytes in each organ. Therefore, I produced Mj-Intgα anti-serum to see if Mj-Intgα can be used as a marker for phagocytic hemocytes.

In immunostaining, the anti-serum bound to Mj-Intgα on the cell surface of hemocytes, because the signals were detected without any permeabilization process. On EF-Hem, almost all hemocytes were positive for Mj-Intgα (Fig. 5B, D), although I also found some Mj-Intgα negative FMB+ hemocytes (indicated by green arrows in Fig. 5H). This suggests that almost all phagocytic hemocytes (but not all) have Mj-Intgα. On T-Hem about 70% of hemocytes were positive for Mj-Intgα. However, the maximum ratio of FMB+ hemocytes in this study was about 10%, suggesting that another 60% of the hemocytes have the ability for phagocytosis. These results also suggest that phagocytic hemocytes could be divided into two types, mainly Mj-Intgα-positive hemocytes and a few Mj-Intgα-negative hemocytes.

A possible lectin receptor in the shrimp *F. chinensis* was identified as an integrin that involved phagocytosis (Wang *et al.,* 2014). The integrin α of silkworm *Bombyx mori* is reported to be granulocyte-specific (Zhang *et al.,* 2014). In kuruma shrimp, strong phagocytic activity was observed in SGC and GC (Kondo *et al.,* 1992). Integrin alpha 5 of *F. chinensis* is homologous to Mj-Intgα, and is reported to interact with white spot syndrome

virus (WSSV) (Sun *et al.,* 2014). WSSV also infects SGC and GC but not HC (Wang *et al.,* 2002). The present results, together with previous results, indicate that Mj-Intgα could be a marker of phagocytic SGCs and GCs.

However, our attempts to isolate Mj-Intgα-positive hemocytes from total circulating hemocyte using this anti-serum by antibody with techniques such as MACS were unsuccessful. Almost all antibody-based isolation techniques use monoclonal antibodies rather than polyclonal antibodies because of their high affinity and specificity. Therefore, a monoclonal antibody that recognizes Mj-Intgα is needed to isolate and further characterize phagocytic hemocytes.

In the summary, I enriched phagocytic hemocytes by a low-cost technique and then conducted RNA-seq analysis between total circulating hemocytes and enriched phagocytic hemocytes. In this way, I identified Mj-Intgα as a gene that was highly expressed in phagocytic hemocytes and cloned it. Anti-serum against rMj-Intgα bound to almost all (but not all) phagocytic hemocytes, suggesting that *M. japonicas* has at least two types of phagocytic hemocytes. Further studies using Mj-Intgα should further clarify the immune system of kuruma shrimp.

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Table 1. Primer sequences used in this study

Primer name	Sequence (5'-3')	Usage
Mj-Intgα_F1	GAC AAG CGG ACC CGA GGT GCA	Cloning
Mj-Intgα_R1	TAG CCT CTG TAC CGG TGG TC	Cloning
Mj-Intgα_F2	GAC CAC CGG TAC AGA GGC TA	Cloning
Mj-Intgα_R2	AAC GTC AGG GTA ACC ATT GC	Cloning
Mj-Intgα_F3	GCA ATG GTT ACC CTG ACG TT	Cloning
Mj-Intgα_R3	AAG CGA TCC TCC TCT TGT CGT TC	Cloning
Mj-Intgα detection_F	GCA ATG GTT ACC CTG ACG TT	RT-PCR
Mj-Intgα detection_R	AGG AAG CGA GGG TAG GAG AG	RT-PCR
EF-1α detection_F	ATG GTT GTC AAC TTT GCC CC	RT-PCR
EF-1α detection_R	TTG ACC TCC TTG ATC ACA CC	RT-PCR
Mj-Intgα_F_NdeI	AAA CAT ATG TTC AAC CTG GAC ACA CAG CAC	Recombinant plasmid
Mj-Intgα_R_NotI	TTT GCG GCC GCC TCG ACC GCC AGC ACC GGC GCC GA	Recombinant plasmid
MjIntgα_qPCR_F	GAC GAG CCA AGC CAT CTG A	qRT-PCR
MjIntgα_qPCR_R	TCC GTC GAG CAG TCT TCA TG	qRT-PCR
DN9598_c0_g1_qPCR_F	ACC CGA CGA TGC CAG AAC	qRT-PCR
DN9598_c0_g1_qPCR_R	TGG GAA GAT TTG GGA TAA GAA GAC	qRT-PCR
DN10184_c0_g1_qPCR_F	GAG TCA GAA GTC GCC GAG TGT	qRT-PCR
DN10184_c0_g1_qPCR_R	TGG CTC AGC AGG TCG TTT AA	qRT-PCR
EF-1α_qPCR_F	ATT GCC ACA CCG CTC ACA	qRT-PCR
EF-1α_qPCR_R	TCG ATC TTG GTC AGC AGT TCA	qRT-PCR

Table 2. Differentially expressed transcripts between T- and EP-Hem

Contig no.	Log FC	Log CPM	FDR	Top hit of homology genes of Penaeidae by Blastx			
				Gene name [Species]	Identity (%)	E-value	GenBank accession no.
DN8588_c0_g1	4.50	5.89	6.04E-07	Integrin alpha 5 [*Fenneropenaeus chinensis*]	30	4.00E-08	AHH32888.1
DN10184_c0_g1	-4.59	9.88	2.31E-09	Hemocyte transglutaminase [*Marsupenaeus japonicus*]	100	0	ABD92928.1
DN9598_c0_g1	-3.91	7.58	3.91E-07	Prophenoloxidase activating enzyme [*Litopenaeus vannamei*]	85	0	AFW98991.1
DN10109_c0_g1	-3.58	8.29	1.62E-06	Pancreatic lipase [*Litopenaeus vannamei*]	29	5.2	AHM88206.1
DN10529_c1_g8	-4.25	5.83	1.86E-06	Alpha 2-macroglobulin [*Fenneropenaeus indicus*]	31	9.5	ADT91769.1
DN9657_c1_g1	-3.35	12.64	2.82E-06	Hemocyte kazal-type proteinase inhibitor [*Penaeus monodon*]	77	1.00E-142	AAP92779.1
DN10956_c3_g7	-3.29	14.05	4.01E-06	Alpha 2-macroglobulin homolog [*Marsupenaeus japonicus*]	99	0	BAC99073.1
DN8333_c0_g1	-3.66	6.56	5.50E-06	Prophenoloxidase activating factor [*Penaeus monodon*]	51	1.00E-73	ABE03741.1
DN5567_c0_g1	-3.50	6.82	5.90E-06	Prophenoloxidase activating factor [*Penaeus monodon*]	86	3.00E-06	ABE03741.1
DN10773_c0_g1	-3.63	6.54	6.26E-06	Cysteine sulfinic acid decarboxylase [*Litopenaeus vannamei*]	45	6.00E-142	AKS04548.1
DN10856_c0_g1	-3.46	6.79	7.36E-06	Alpha glucosidase [*Litopenaeus vannamei*]	44	0	CAB85963.1
DN7484_c0_g1	-3.44	6.70	8.56E-06	Chitinase [*Marsupenaeus japonicus*]	44	9.00E-04	BAA12287.1

Log2-transformed fold change (FC), log2-transformed counts per million (CPM), and false discovery rate (FDR) refer to the differential expression analysis between T- and EP-Hem. Log FC, when positive, indicates transcripts that are expressed more highly in EP-Hem and when negative, indicates transcripts that are expressed more highly in T-Hem.

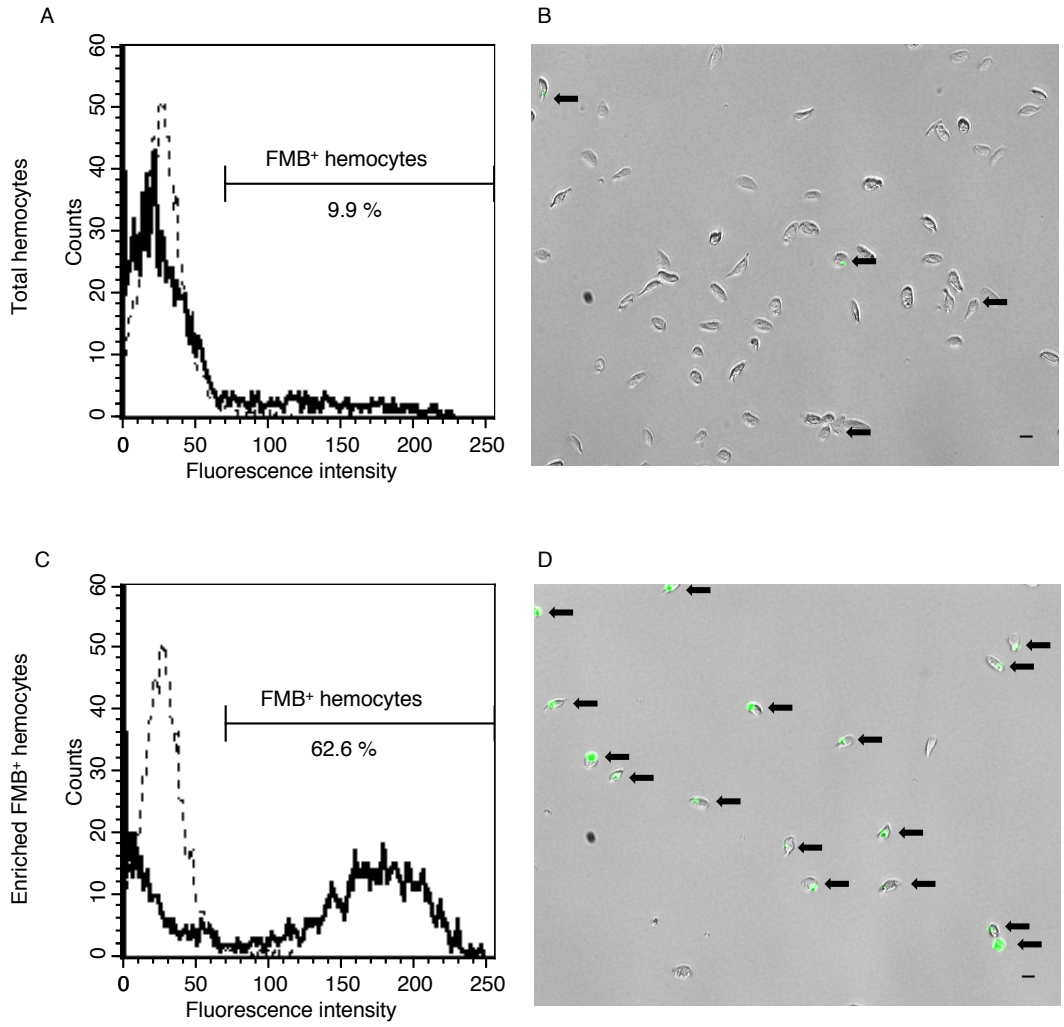

Figure 1. Flow cytometry analysis and fluorescence microscopy observation of total and enriched FMB+ hemocytes from a shrimp. FCM analysis of T-Hem (A) and EF-Hem (C). Dotted line indicates negative control of FL-1 value. Fluorescence microscopy observation of T-Hem (B) and EF-Hem (D). Green colors in picture B and D came from fluorescence of FMB. Black arrow indicates hemocyte engulfed FMB. Black bar indicates 10 μm scale.

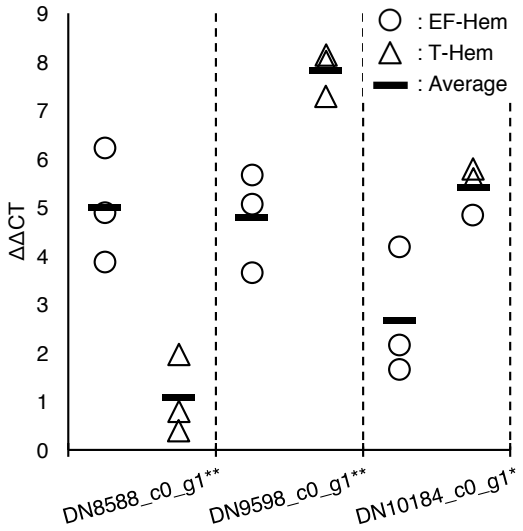

Figure 2. qRT-PCR analysis of three transcripts (DN8588_c0_g1, DN9598_c0_g1 and DN10184_c0_g1). ΔCt values analyzed by qRT-PCR. Higher ΔCT value indicates higher expression. Double asterisk (**) on the name of each transcript indicates p-value less than 0.01 and single asterisk (*) indicates p-value less than 0.05.

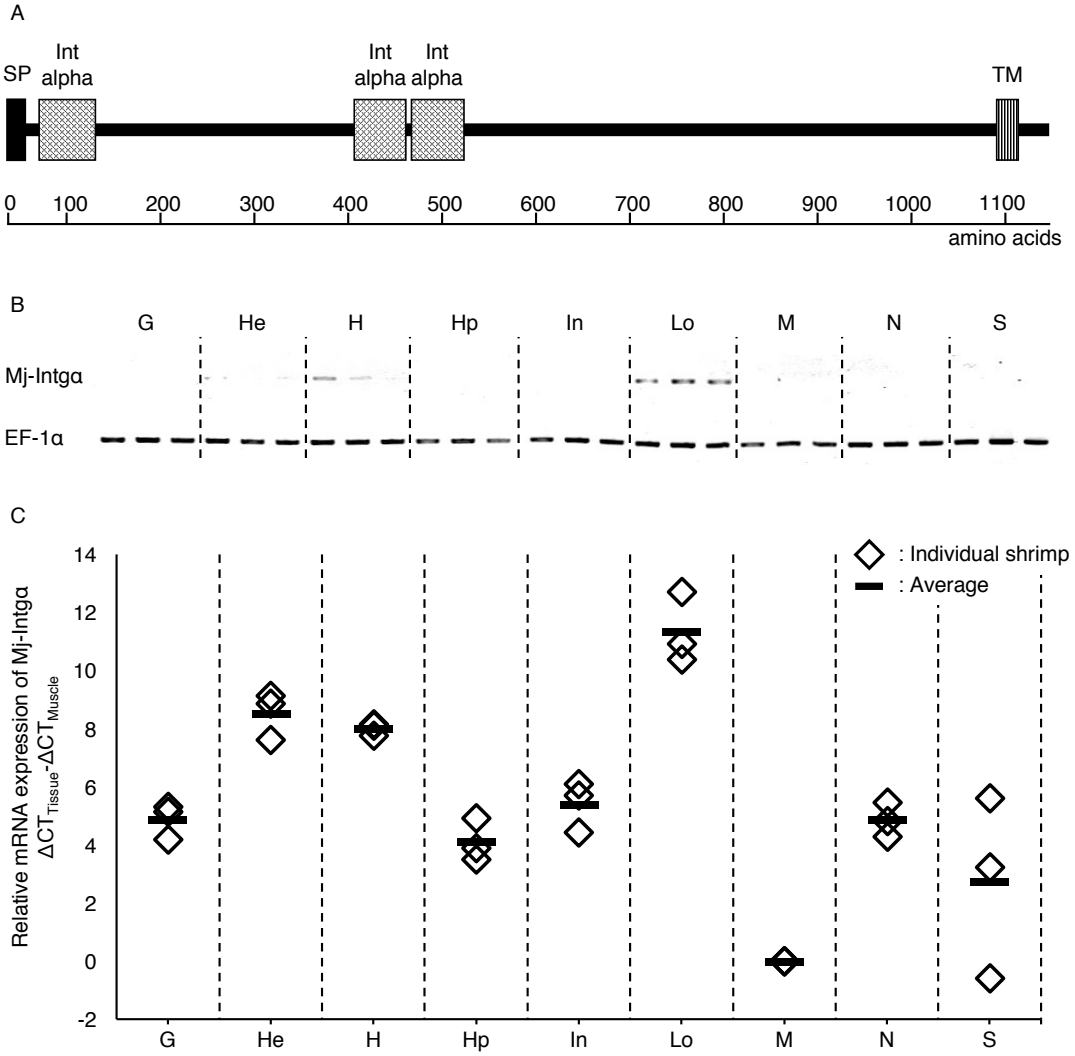

Figure 3. Molecular characterization and tissue distribution of Mj-Intgα. Predicted domain structures of Mj-Intgα (A): Signal peptide (SP), integrin (Int) alpha domain and transmembrane (TM) site. Tissue distribution of Mj-Intgα by RT-PCR (B) and qRT-PCR (C) using Gills (G), heart (He), hemocytes (H), hepatopancreas (Hp), intestine (In), lymphoid organ (Lo), muscle (M), nerve (N) and stomach (S).

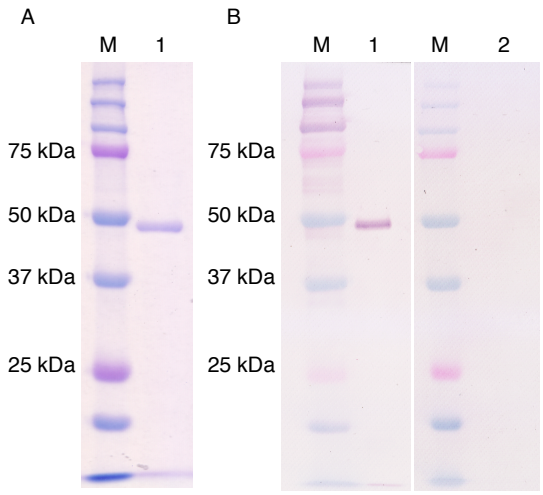

Figure 4. Purity of rMj-Intgα and reactivity of anti-rMj-Intgα serum against rMj-Intgα. SDS-PAGE analysis of rMj-Intgα (A) and western-blotting analysis against rMj-Intgα (B) using anti-rMj-Intgα serum (Lane 1) and non-immunized rabbit serum (Lane 2). Marker (Lane M).

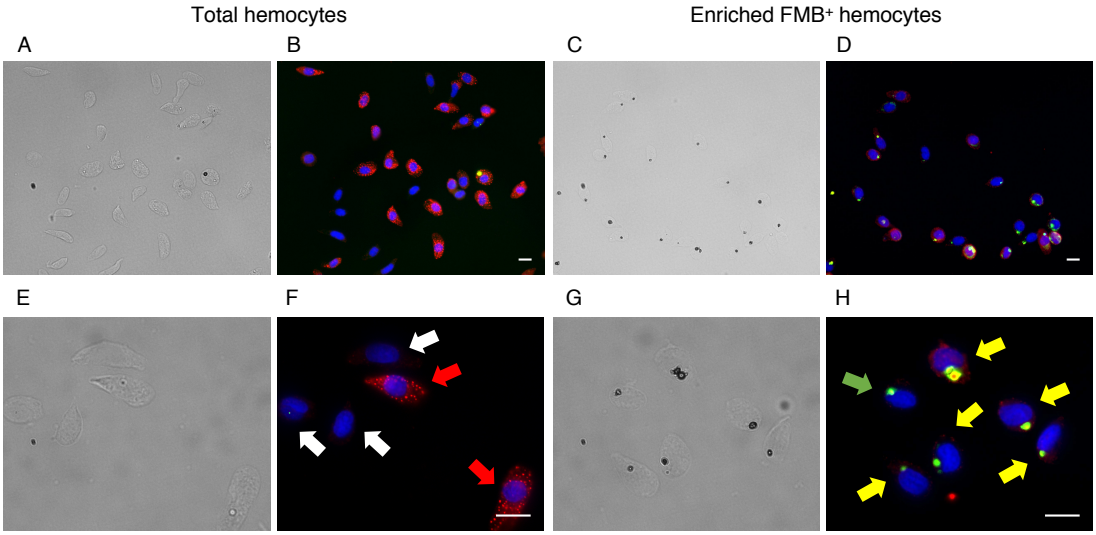

Figure 5. Immunostaining of Mj-Intgα on of total and enriched FMB+ hemocytes from a shrimp. White arrow, Mj-Intgα- hemocytes; red arrow, Mj-Intgα+ hemocytes; yellow arrow, Mj-Intgα+ FMB+ hemocytes; green arrow, Mj-Intgα- FMB+ hemocytes. Scale bars, 10 μm.

第４章　Isolation and molecular characterization of hemocyte sub-populations in kuruma shrimp *Marsupenaeus japonicus*

第１節 Abstract

Crustacean hemocytes, which have usually been classified morphologically based on dyeing methods such as Giemsa or May-Giemsa staining, have recently been categorized with monoclonal antibodies or marker genes. However, these techniques have not become widely used, resulting in the use of different classification methods for hemocytes among laboratories. Therefore, in this research, I aimed to develop a classification method that can be widely used. The method uses lectins and a magnetic-activated cell sorting system (MACS system) to isolate sub-populations. Two lectins, wheat germ agglutinin (WGA) and tomato lectin (*Lycopersicon esculentum* lectin: LEL), characteristically bound to the hemocytes, which allowed them to be classified into three sub-populations. Furthermore, by using LEL and the MACS system, different sub-populations of hemocyte could be isolated. These sub-populations were characterized as non-granular and granular hemocytes, and the accumulation patterns of the gene transcripts were consistent with the results of a functional analysis reported previously. The lectin-based hemocyte isolation method developed in this study has good reproducibility.

第２節 Introduction

Hemocytes of shrimp act as immune organs (for reviews see Jiravanichpaisal *et al.*, 2006; Soderhall, 2016; Tassanakajon *et al.*, 2013). The classification of hemocytes is indispensable to analyze the biological defense mechanism in detail. So far, dyeing methods such as Giemsa or May-Giemsa staining, and antibody-based classification methods have been developed based on the leukocyte classification methods of mammals. The Giemsa or May-Giemsa staining method is excellent for staining the cytoplasmic granules of hemocytes, which contain anti-microbial peptides (Bachère *et al.*, 2004; Rosa and Barracco 2010). Hemocytes can be roughly divided into three types morphologically, hyaline hemocytes (HCs), semi-granular hemocytes (SGCs) and granular hemocytes (GCs) by Giemsa or May-Giemsa staining (Johansson *et al.*, 2000; Söderhäll and Smith, 1983). However, the results of Giemsa and May-Giemsa staining are not always the same, and can be affected by pH, dyeing time, humidity and worker's degree of training. Therefore, these methods are not well-suited for quantitative experiments.

Rodriguez *et al.* (1995) used whole hemocytes of kuruma shrimp (*Marsupenaeus japonicus*) as antigens and produced ten kinds of monoclonal antibodies. Sung *et al.* (1999) and Sung and Sun (2002) used whole hemocytes of black tiger shrimp (*Penaeus monodon*) as antigens to produce four monoclonal antibodies. Similarly, van de Braak *et al.* (2000) and Winotaphan *et al.* (2005) produced four types of monoclonal antibodies using black tiger shrimp hemocytes or hemocyte lysate as antigens. As a result of immunological staining using these monoclonal antibodies, even the same morphologically classified cells such as HCs, SGCs and GCs showed differences in reactivity to their cell surface antigens, and due

to the reactivity difference of the monoclonal antibodies, hemocytes have been defined in more detail. More recently, monoclonal antibodies reactive to *Litopenaeus vannamei* hemocytes were developed by Lin *et al.* (2007) and Zhan *et al.* (2008). Using these antibodies Xing *et al.* (2017) succeeded in isolating two sub-populations of hemocytes: agranulocytes and granulocytes. However, these monoclonal antibodies are not widely used for classifying shrimp hemocytes because it is difficult to prepare identical monoclonal antibody-producing clones in different laboratories and because few suppliers are interested in developing products for crustaceans due to the small number of researchers.

Therefore, it is also important to classify specific hemocytes without relying on antibodies. In other organisms especially in human, cells are classified based on sugar chains present on the cell surface. Lectins are proteins that bind to sugar chains, and are used for staining and classification of various cells, such as cancer cells, based on their sugar chains (Christiansen *et al.*, 2014; Gabius *et al.*, 2015; Kobata 1992). Until now, hemocytes of bees (*Apis mellifera*), fly (*Drosophila melanogaster*), mosquito (*Anopheles gambiae*), Pacific oyster (*Crassostrea gigas*) and Europe mussel (*Mytilus edulis*) have been classified by lectins (Jiang *et al.*, 2016; Marringa *et al.*, 2014; Pipe 1990; Rodrigues *et al.*, 2010; Tirouvanziam *et al*., 2004). In addition, cytoplasmic granules of hemocytes of ridgeback prawn (*Sicyonia ingentis*) and American lobster (*Homarus americanus*) have been reported to be stained by wheat germ agglutinin (WGA) (Martin *et al.*, 2003). Furthermore, WGA, tomato lectin (*Lycopersicon esculentum* lectin: LEL) and peanut agglutinin (PNA) were found to bind to some of the GCs, SGCs and HCs of *L. vannamei* (Estrada *et al.,* 2016). However, few studies have stained shrimp hemocytes with lectins, and molecular biological analyses of lectin-positive

hemocytes have not been conducted.

In this study, I isolated two hemocyte sub-populations using LEL and a magnetic-activated cell sorting system (MACS system), and then predicted their functions by measuring the accumulation of mRNA transcripts by RNA-seq and quantitative RT-PCT (qRT-PCR) analyses.

第３節 Material and methods

Shrimp samples

Apparently healthy kuruma shrimp (*Marsupenaeus japonicus*) were obtained from farms in Okinawa and Miyazaki prefecture, Japan. Shrimps were kept in tanks provided with a water recirculating system maintained at 25 ^{0}C and 30-35 ppt. Shrimps were acclimatized for at least 3 days before the experiment.

Lectin staining of hemocytes by LEL and WGA

One (1) mL of hemolymph was collected from each shrimp using a 23-gauge needle and syringe containing 1 mL of anti-coagulant (0.45 mM NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH: 5.6) (Söderhäll and Smith, 1983), and then centrifuged to obtain hemocytes. The hemocytes were fixed with 4% paraformaldehyde (PFA) in PBS (137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH: 7.3) for 15 min at room temperature. One of two lectins, DyLight 488-conjugated LEL or FITC-conjugated WGA (both Vector Laboratories, Inc., USA), was added at a ratio of 2 μg to 10^6 fixed cells and reacted for 15 minutes at 4 ^{0}C in reaction buffer (0.5% BSA, 2 mM EDTA in PBS). After washing twice, hemocytes were analyzed by flow cytometry and observed under a fluorescence microscope. The fluorescent intensities of at least 5,000 DyLight 488- or FITC-stained hemocytes were analyzed by FACSCalibur (Becton-Dickinson, USA) using an FL-1 filter with Cell Quest Pro software ver. 5.2.1 (Becton-Dickinson, USA). Simultaneously relative cell size and relative cell complexity were determined by FACSCalibur and Cell Quest Pro software ver. 5.2.1 using a forward-scatter (FSC) filter and

a side-scatter (SSC) filter, respectively. The stained hemocytes were examined by bright- and fluorescent-field using upright microscope ELIPSE Ci (Nikon Co., Japan), and the images were analyzed by NIS-Elements (Nikon Co.) and ImageJ ver. 2.0.0. (Schneider et al., 2012). The assay was performed three times from three individual shrimps.

Double lectin staining

PFA-fixed hemocytes were prepared as described above. Both biotin-conjugated LEL (Vector Laboratories, Inc.) and FITC-conjugated WGA were added at a ratio of 2 µg each to 10^6 fixed cells and reacted for 15 minutes at 4 ^{0}C in reaction buffer. After the hemocytes were washed twice, DyLight 550-conjugated natural streptavidin protein (Abcam plc., U.K.) was added at a ratio of 0.4 µg to 10^6 fixed cells and reacted for 15 minutes at 4 ^{0}C in reaction buffer. After washing twice, hemocytes were examined by bright- and fluorescent-field as described above. The assay was performed three times from three individual shrimps.

Isolation of LEL^{Dim} and LEL^{Strong} hemocytes by MACS system

PFA-fixed hemocytes were prepared as described above. For isolation of LEL^{Dim}, hemocytes were stained with biotin-conjugated LEL (Vector Laboratories, Inc.) at a ratio 1 µg to 10^6 fixed cells for 15 minutes at 4 ^{0}C in reaction buffer. After washing once, hemocytes were reacted with 10 µL of streptavidin microbeads (Miltenyi Biotec, Germany) in 90 µL of reaction buffer for 15 min at 4 ^{0}C. After washing once, hemocytes were separated by MACS using MS column (Miltenyi Biotec) and MiniMACS separator (Miltenyi Biotec) following the manufacturer's protocol. The negative fraction was collected as LEL^{Dim} hemocytes. For

isolation of LEL^{Strong}, hemocytes were stained with biotin-conjugated LEL at a ratio 0.1 µg to 10^6 fixed cells for 15 minutes at 4 ^{0}C in reaction buffer. After washing once, hemocytes were reacted with 1 µL of streptavidin microbeads in 99 µL of reaction buffer for 15 min at 4 ^{0}C. After washing once, hemocytes were separated by MACS. The positive fraction was collected as LEL^{Strong} hemocytes.

Total, LEL^{Dim} and LEL^{Strong} hemocytes were analyzed by flow cytometry. Five thousand (5,000) events of each sample were collected and then FSC and SSC analyses were conducted by FACSCalibur with Cell Quest Pro software ver. 5.2.1 as described above. Two gates, R1 and R2, were established based on the FSC and SSC, and the percentage of dot plots in each gate were analyzed by Cell Quest Pro software. The assay was performed six times.

May-Giemsa staining of LEL^{Dim} and LEL^{Strong} hemocytes

LEL^{Dim} and LEL^{Strong} hemocytes were collected as described above. Each hemocyte suspension was spread on a glass slide in a cell collection bucket SC-2 (TOMY, Japan) at 100 x g for 1 min. Glass slides were dried, stained for 3 min with 20% May-Grunwald stain solution (Wako, Japan) in 0.67 mM phosphate buffer (pH: 6.6), washed with phosphate buffer, stained for 15 min with 4% Giemsa stain solution (Wako) in 0.67 mM phosphate buffer (pH: 6.6), washed with tap water, dried, mounted with Malinol (Muto Pure Chemicals, Japan) and visualized with NIS-Elements software.

cDNA Library construction and RNA sequencing by Illumina Miseq

Total, LEL^{Dim} and LEL^{Strong} hemocytes were collected from six shrimps as described above. The PFA-fixed hemocytes were digested with proteinase K (Masuda *et al.*, 1999). Total RNA was then extracted with a NucleoSpin® RNA XS kit (Takara Bio Inc., Japan) following the manufacturer's protocol. The total RNAs of each type of hemocyte were pooled. The concentration and purity of total RNA were measured using a Qubit® RNA HS Assay Kit and NanoDrop Lite (both Thermo Fisher Scientific Inc., USA). cDNA libraries were prepared with total RNA using a TruSeq stranded mRNA sample preparation kit (Illumina Inc., USA) following the manufacturer's protocol. The libraries were amplified with 20 cycles of PCR and contained indexes within the adaptors. The yields in the amplified libraries were measured with a Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific Inc.) and D1000 ScreenTape System (Agilent Technologies, USA). The yields of LEL^{Dim}, LEL^{Strong} and total hemocytes were 1.9, 0.184 and 14.5 ng/μL, respectively, with average lengths of 293, 286 and 297 bp, respectively, indicating concentrations 10.3, 1.05 and 77.5 nM, respectively. Six (6) pmol of each library was sequenced using MiSeq (Illumina Inc.) and MiSeq reagent kit version 2 (Illumina Inc.) with 75 nt paired end reads.

***De novo* assembly and identification of differentially expressed transcripts**

The reads were assembled by Trinity v2.5.1 (Grabherr *et al.*, 2011) using default parameters (minimum assembled transcripts length 200) to obtain trinity-assembled transcripts. The sequenced libraries were mapped back to the reference trinity-assembled transcripts using RSEM (Li and Dewey, 2011) to quantify the read counts. Read counts were

normalized by trimmed mean of M-values (TMM) to account for differences in library size (Robinson and Oshlack, 2010) and then normalized by transcripts per million (TPM) to account for differences in transcript length. The differentially expressed transcripts between total, LEL^{Dim} and LEL^{Strong} hemocytes libraries were identified using EdgeR (Robinson *et al.*, 2010) including a p-value cutoff for false discovery rate of 0.001 and a minimum 16-fold change in expression. Blastx program (Altschul *et al.*, 1997) was then used for homologous gene searching with an e-value cut-off of 0.05 in Penaeidae's 5,942 proteins (downloaded at Oct. 18, 2018) in NCBI database (http://www.ncbi. nlm.nih.gov).

Transcripts quantification of immure-related genes by qRT-PCR

Total, LEL^{Dim} and LEL^{Strong} hemocytes were extracted from three shrimps, then total RNAs were extracted as described above. cDNAs were synthesized from RNA of each sample using a High capacity cDNA reverse transcription kit (Thermo Fisher Scientific Inc.). After synthesis, cDNA samples were diluted five times with distilled water and 2 µl of samples were used for qRT-PCR. The set of primers were designed based on registered sequences or trinity-transcripts (Table 1). Elongation factor 1α (EF-1α: as an internal control) for qRT-PCR (Table 1). qRT-PCR was conducted using THUNDERBIRD SYBR qPCR Mix (TOYOBO Co. Ltd., Japan) and condition was 95 ^{0}C for 1 min, 40 cycles of 95 ^{0}C for 15 secs and 60 ^{0}C for 1 min followed by dissociation analysis step. mRNA accumulation of each gene was calculated as ΔCT by comparing with CT value of EF-1α (as a reference gene). The statistical significance between total, LEL^{Dim} and LEL^{Strong} hemocytes respectively was analyzed using t-test.

Lectin staining on hemocytes phagocyted micro beads

Shrimps were injected with 200 μL of 10% suspension of fluorescent beads (Fluoresbrite YO Cartoxylate Microspheres 1.0 μm: Polysciences, Inc., USA) in artificial seawater. Three (3) hours post injection, PFA-fixed hemocytes were prepared and stained by DyLight 488-conjugated LEL or FITC-conjugated WGA, respectively as described above. The stained hemocytes were examined by bright- and fluorescent-field as described above. The assay was performed three times from three individual shrimps.

第４節 Results

Lectin staining of total hemocytes

Both LEL and WGA showed reactivity to all hemocytes, however there were a difference in reactivity, and they could be classified into two subpopulations, LEL^{Dim}/LEL^{Strong} and WGA^{Dim}/WGA^{Strong}, respectively (Fig. 1 and 2). LEL reacted strongly with cells with relatively small and simple intracellular structure (Fig. 1D), whereas WGA reacted strongly with cells with relatively large and complex intracellular structure (Fig. 2D). LEL and WGA strongly reacted with the cell surface and the intracellular structure of hemocytes, respectively (Fig 3). Dim-positive and strong-positive of each lectin hemocytes were also observed under fluorescent-field (Fig 3).

Double lectin staining

Double lectin staining of total hemocytes by LEL and WGA was able to divide hemocytes into three sub-populations: LEL-positive, WGA-positive and LEL/WGA-positive hemocytes (Fig. 4). The ratio of LEL/WGA-positive hemocytes was 19% (n=3), and the fluorescent intensity of LEL/WGA-positive hemocytes was weaker than the ratios of other sub-populations. As with single staining, LEL well stained the cell surface and WGA well stained the intracellular structure of hemocytes.

Isolation of LEL^{Dim} and LEL^{Strong} hemocytes by MACS system

Using the MACS system and biotin-conjugated LEL, LEL^{Dim} hemocytes and LEL^{Strong} hemocytes were isolated, respectively. May-Giemsa staining showed that LEL^{Dim} hemocytes

(Fig. 5C) were relatively larger than LEL^{Strong} hemocytes (Fig. 5D), and unlike the latter, contained intracellular granules and a large cytoplasm compared to the nucleus. Regions 1 and 2 before separation of hemocytes were 45.8 ± 12.4% and 51.9 ± 12.0%, respectively, whereas after separation of LEL^{Dim} hemocytes, they were 11.0 ± 3.2% and 83.8 ± 6.0%, and after separation of LEL^{Strong} hemocytes, they were 86.7 ± 7.2% and 10.9 ± 6.6% (n=6) (Fig. 6).

Differentially expressed transcripts by RNA sequencing

The assembled transcripts contained 11,870 trinity-transcripts. The median trinity-transcripts length was 339 bp and the N50 (weighted median) was 539 bp. I identified 2,630 differentially expressed transcripts based on a p-value cut-off for FDR of 0.001 and a minimum 16-fold change in expression. In blastx searches, 163 trinity-transcripts matched Penaeidae proteins with e-values less than 0.05 (Table 2), 31 of which were immune-related (Fig. 7). The immune-related trinity-transcripts fell into four clusters that were highly expressed in (1) only LEL^{Strong} hemocytes, (2) both total and LEL^{Dim} hemocytes, (3) only total hemocytes and (4) only LEL^{Dim} hemocytes (Fig. 7).

Differentially expressed transcripts by qRT-PCR

In the qRT-PCR results, the ΔCt values of transcripts of two major anti-microbial peptides (AMPs) (crustin and penaeidin-II) and c-type lysozyme were lower in LEL^{Strong} hemocytes than in total hemocytes, while the ΔCt values of transcripts of hemocyte transglutaminase and prophenoloxidase (proPO) activation enzyme were lower in LEL^{Dim} hemocytes than in

total hemocytes (Fig. 8). The trend was also seen in that the ΔCt values of transcripts of Toll and integrin α were lower in LEL^{Strong} hemocytes than in total and LEL^{Dim} hemocytes (Fig. 8)..

Lectin staining of hemocytes phagocyted micro beads

The fraction of hemocytes phagocyted micro beads was 5.6% (n=3). Both LEL-positive and -negative hemocytes phagocyted micro beads (Fig. 9B, D), whereas only WGA-positive hemocytes phagocyted micro beads (Fig. 10B, D). In addition, the fluorescent intensity of WGA-positive beads phagocyted hemocytes tended to be weaker than other WGA-positive hemocytes.

第５節 Discussion

The stainability of hemocytes by two lectins, WGA and LEL, were different. This suggests that sugar chains on hemocytes are different depending on the type of hemocytes. Like the reports of Martin *et al.*, (2003) and Estrada *et al.*, (2016), WGA strongly stained the granules of hemocytes. The flow cytometry data also showed a strong WGA signal in hemocytes with high SSC values, suggesting that WGA stains granules of hemocytes. The investigation of the existence of granules on hemocytes is important for characterization of hemocytes. However, it was unclear which hemocytes contained granules on dyeing methods such as Giemsa or May-Giemsa staining. Combination of WGA staining, microscopic observation and FCM analysis, it became easier to prove the existence of granules on hemocytes. In contrast to WGA, LEL appeared to bind to the cell surface and not cytoplasmic granules. Since LEL stained the cell surface, MACS system could be used.

May-Giemsa staining showed that LEL^{Dim} hemocytes contained a lot of cytoplasmic granules, while LEL^{Strong} hemocytes contained little or no granules. The flow cytometry data also showed that LEL^{Strong} hemocytes was smaller and had lower SSC value than LEL^{Dim} hemocytes. These results indicate that hemocytes could be divided into two sub-populations: LEL^{Strong} hemocytes that were agranulocytic and LEL^{Dim} hemocytes that were granulocytic.

The two populations were associated with specific transcripts. Transcripts of hemocyte transglutaminase, which is related to clotting of hemolymph (Maningas *et al.*, 2013), were highly accumulated in LEL^{Strong} hemocytes in both the RNA-seq and qRT-PCR analyses.

Abundant transglutaminase transcripts were also reported on HCs (also called agranular hemocytes) in *L. vannamei* (Yang *et al.*, 2015). The transglutamase results also strongly suggest that LEL^{Strong} (i.e., agranular) hemocytes contribute to blood coagulation in kuruma shrimp. On the other hand, total and LEL^{Dim} hemocytes highly accumulated transcripts of crustin, crustin-like, penaeidin-II and c-type lysozyme, as shown by the RNA-seq and qRT-PCR analyses. AMPs and c-type lysozyme are also present in cytoplasmic granules of hemocytes (Bachère *et al.*, 2004; Rosa and Barracco 2010). Our RNA-seq analysis also showed that LEL^{Dim} hemocytes had abundant transcripts of proPO activation enzymes and serine proteases, which are also proPO-related enzymes (Cerenius and Söderhäll 2004; Hernández-López *et al.*, 1996). In many crustaceans, the proPO system is carried by granular hemocytes (Söderhäll 2016; Sung *et al.*, 1998; Yang *et al.*, 2015). Based on these previous reports and the present results, LEL^{Dim} hemocytes (i.e. granulocytes) are responsible for the production of AMPs and c-type lysozyme, and contribute to the proPO system, as reported previously.

The hemocytes which have the phagocytic activity vary greatly from species to species in crustacean. In kuruma shrimp, strong phagocytic activity was observed in SGCs and GCs (Kondo *et al.*, 1992). LEL^{Dim} hemocytes accumulate transcripts involved in foreign object recognition, such as integrin, lectins, toll and scavenger receptor (Art *et al.*, 2007; Bi *et al.*, 2015; Lin *et al.*, 2013; Wang *et al.*, 2010; Wang and Wang, 2013; Wang *et al.*, 2014; Yang *et al.*, 2007; Zhang *et al.*, 2012). Furthermore, there was a correlation between WGA-positive hemocytes and phagocytosis, not LEL-positive hemocytes (Fig. 10B, D) in this study. Together, these results indicate that kuruma shrimp granular hemocytes are the

main players in phagocytosis. Interestingly, LEL-positive not WGA-positive cells were reported to be phagocytotic in Pacific oyster *C. gigas* (Jiang *et al.*, 2016), which suggests that the composition and function of cell surface glycans can differ in the same invertebrates.

Some hemocytes stained with both WGA and LEL. Lin and Söderhäll (2011) argue that GCs and SGCs differentiate from HCs. In this study, both LEL- and WGA-positive hemocytes were present, but we were unable to analyze their functions. For example, both LEL- and WGA-positive hemocytes may be in transition from HCs to GCs or SGCs. By using a combination of LEL and WGA, it is now possible to more accurately classify the types, functions and life cycles of hemocytes.

Since my lectin-based hemocyte isolation method requires cell fixation, functional analysis was impossible. Therefore, further studies are needed to identify buffers that can make it possible to stain living hemocytes with lectins to conduct functional analysis or extract high quality RNAs. It is also necessary to identify the antigens of LEL and WGA to clarify how hemocytes are classified. Despite these problems, lectin-based hemocyte isolation uses easily available lectins and a relatively inexpensive MACS system, which should make it useful in many laboratories.

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Table 1. Primer sequences used in this study

Primer name	Sequence (5'-3')	Accession number
EF-1α_qRT-PCR_F	ATT GCC ACA CCG CTC ACA	AB458256.1
EF-1α_qRT-PCR_R	TCG ATC TTG GTC AGC AGT TCA	
Crustin_qRT-PCR_F	AAC TAC TGC TGC GAA AGG TCT CA	AB121740-4.1
Crustin_qRT-PCR_R	GGC AGT CCA GTG GCT TGG TA	
Penaeidin-II_qRT-PCR_F	TTA GCC TTA CTC TGT CAA GTG TAC GCC	KU057370.1
Penaeidin-II_qRT-PCR_R	AAC CTG AAG TTC CGT AGG AGC CA	
C-type lysozyme_qRT-PCR_F	ATT ACG GCC GCT CTG AGG TGC	AB080238.1
C-type lysozyme_qRT-PCR_R	CCA GCA ATC GGC CAT GTA GC	
Anti-lipopolysaccharide factor_qRT-PCR_F	AGC CTC CTT TTC CTT TCC CCT	KX424931.1
Anti-lipopolysaccharide factor_qRT-PCR_R	CAC AAT CCT GTC AGT TTT TCC GC	
C-type lectin_qRT-PCR_F	ACG CTG GTG TGA TGC CCG	KJ175168.1
C-type lectin_qRT-PCR_R	ACC GAG TCT GAG CCG CCT AA	
Hemocyte Transglutaminase_qRT-PCR_F	GAG TCA GAA GTC GCC GAG TGT	DQ436474.1
Hemocyte Transglutaminase_qRT-PCR_R	TGG CTC AGC AGG TCG TTT AA	
Transglutaminase_qRT-PCR_F	TGA CTG CGA AGA ACA TGA GC	AB162767.1
Transglutaminase_qRT-PCR_R	GTT CTT GGT TTC CCC GAC TC	
Prophenoloxidase activation enzyme_qRT-PCR_F	ACC CGA CGA TGC CAG AAC	This study
Prophenoloxidase activation enzyme_qRT-PCR_R	TGG GAA GAT TTG GGA TAA GAA GAC	
Prophenoloxidase activation factor_qRT-PCR_F	TCA AGG AGG TGG CTC TCC CT	This study
Prophenoloxidase activation factor_qRT-PCR_R	GAT ACC CGA ACC CGG TCT CC	
Prophenoloxidase_qRT-PCR_F	CCG AGT TTT GTG GAG GTG TT	AB073223.1
Prophenoloxidase_qRT-PCR_R	GAG AAC TCC AGT CCG TGC TC	
Toll_qRT-PCR_F	ACT GGA ACG TGT TGG GAA GA	AB333779.1
Toll_qRT-PCR_R	TGC AAG TCC AGA ACC TCC AA	
Integrin α_qRT-PCR_F	GAC GAG CCA AGC CAT CTG A	LC114983.1
Integrin α_qRT-PCR_R	TCC GTC GAG CAG TCT TCA TG	

Table 2. 163 trinity-transcripts were hit by blastx on Penaeidae proteins e-value less than 0.05

	Result of blastx				Log2-transformed TMM-TPM value		
Trinity-transcripts	**Top hit of homology genes, gene name [spicies]**	**Identity (%)**	**E-Value**	**Bit-Score**	**Total**	**LEL^{Dim}**	**LEL^{Strong}**
DN6941_c0_g1	AVG44186.1 crustin-like protein [Penaeus japonicus]	100	1.12E-44	140.0	11.23	9.96	6.80
DN7229_c0_g1	ACP40176.1 crustin Pm5 [Penaeus monodon]	54.545	0.026	30.0	8.35	7.25	-
DN7517_c0_g1	ABD92928.1 hemocyte transglutaminase [Penaeus japonicus]	96.078	4.56E-96	303.0	6.44	-3.27	8.29
DN7410_c2_g1	AJI42795.1 crustacean hematopoietic factor-like protein [Penaeus vannamei]	78.689	1.02E-27	99.8	7.77	3.48	5.99
DN7472_c0_g3	ABV55648.1 Ubc protein, partial [Penaeus monodon]	100	1.75E-41	137.0	7.76	3.74	-
DN7147_c0_g1	AJI42795.1 crustacean hematopoietic factor-like protein [Penaeus vannamei]	41.791	8.23E-11	55.1	7.41	6.95	2.63
DN7399_c0_g2	AXP33454.1 selenium-dependent glutathione peroxidase [Penaeus japonicus]	99.465	3.77E-137	385.0	7.21	6.62	-
DN7398_c8_g1	BAM35674.1 putative reverse transcriptase [Penaeus japonicus]	96.774	1.14E-13	63.5	7.02	6.03	-
DN7337_c0_g1	BAJ78983.1 heat shock protein 90 [Penaeus japonicus]	100	4.26E-21	84.7	6.99	6.53	-
DN7564_c0_g1	ANE31673.1 C-type lectin [Penaeus merguiensis]	78.788	7.53E-76	224.0	6.87	5.78	1.34
DN7561_c3_g1	AOD27416.1 Delta, partial [Penaeus vannamei]	36.842	3.30E-06	45.8	6.80	1.12	6.18
DN1109_c0_g1	AIU99749.1 mitochondrial cytochrome c oxidase subunit VIb [Penaeus vannamei]	95.181	2.39E-56	170.0	6.65	6.53	-0.94
DN7484_c0_g2	BAD34945.1 serine proteinase homologue, partial [Penaeus japonicus]	97.345	0	651.0	6.59	5.31	-
DN7345_c0_g1	ALA09086.1 pacifastin light chain-like serine proteinase inhibitor [Penaeus monodon]	38.043	2.12E-10	57.4	6.50	6.31	-
DN5604_c0_g1	AMQ26208.1 Rac1 [Penaeus vannamei]	100	6.35E-11	52.4	3.24	6.45	-
DN7397_c10_g1	AGV55415.1 innexin 2 [Penaeus monodon]	22.131	1.77E-21	92.8	6.41	5.93	-
DN7451_c1_g2	AID61753.1 fructose 1,6-biphosphate-aldolase A [Penaeus chinensis]	97.808	0	746.0	6.41	6.08	-
DN6409_c0_g1	AEP83534.1 cyclophilin A [Penaeus vannamei]	94.512	7.08E-105	301.0	6.25	5.77	-
DN7462_c0_g1	ARO77488.1 facilitate fatty acid transport protein [Penaeus vannamei]	25.934	3.28E-14	73.6	6.24	6.14	0.31
DN4509_c0_g1	BAM65719.1 myosin heavy chain type 1 [Penaeus monodon]	30.120	3.29E-05	38.5	1.65	-	6.12
DN7008_c0_g1	ABD65308.1 eukaryotic translation initiation factor 3 subunit k, partial [Penaeus vannamei]	95.804	1.01E-101	290.0	5.98	5.84	-
DN7175_c0_g1	AQW41372.1 peroxiredoxin [Penaeus monodon]	98.985	8.96E-146	405.0	5.96	5.69	-
DN5872_c0_g1	ADP30959.1 COP9 constitutive photomorphogenic-like protein subunit 6 [Penaeus monodon]	20.913	1.12E-10	59.3	5.90	5.16	-
DN7357_c0_g2	AFT92034.1 triose-phosphate isomerase [Penaeus vannamei]	98.394	0	513.0	5.89	5.81	-2.14
DN7553_c0_g1	BBC20717.1 integrin alpha ps [Penaeus japonicus]	99.728	0	1441.0	5.87	4.46	0.37
DN7444_c0_g1	BAC57943.1 cathepsin C [Penaeus japonicus]	99.444	2.48E-117	345.0	5.07	5.86	-

Table 2. Continued

	Result of blastx				Log2-transformed TMM-TPM value		
Trinity-transcripts	**Top hit of homology genes, gene name [spicies]**	**Identity (%)**	**E-Value**	**Bit-Score**	**Total**	**LEL^{Dim}**	**LEL^{Strong}**
DN5833_c0_g1	BAA12287.1 chitinase [Penaeus japonicus]	43.902	0.001	38.1	5.76	4.94	-
DN61_c0_g1	ACJ47904.1 nascent polypeptide-associated complex alpha [Penaeus monodon]	98.558	3.81E-112	320.0	5.73	5.25	-
DN9914_c0_g1	ABD92928.1 hemocyte transglutaminase [Penaeus japonicus]	99.517	1.36E-151	436.0	2.20	-	5.67
DN6868_c0_g1	ADR74382.1 prophenoloxidase-activating enzyme 2a [Penaeus monodon]	70.745	0	525.0	5.64	4.23	-
DN6682_c0_g1	ACO59906.1 eukaryotic translation initiation factor 3 subunit G [Penaeus monodon]	98.630	2.8E-178	493.0	5.59	4.70	-
DN7095_c0_g1	AEE25939.1 V-H-ATPase subunit A [Penaeus vannamei]	96.970	5.03E-37	135.0	4.77	5.54	-
DN7484_c0_g1	BAD34945.1 serine proteinase homologue, partial [Penaeus japonicus]	75.964	3.72E-180	503.0	5.43	4.60	-
DN7504_c0_g1	AOZ86849.1 tyrosine-protein phosphatase non-receptor type 2 [Penaeus japonicus]	21.339	1.23E-05	45.1	5.36	4.75	-
DN6898_c0_g1	ANC55855.1 selenoprotein M [Penaeus vannamei]	94.656	9.56E-77	228.0	4.85	5.33	-1.67
DN5050_c0_g1	AIW39898.1 calreticulin [Penaeus monodon]	98.131	4.74E-133	385.0	5.32	5.02	-
DN5464_c0_g1	ADM87522.1 Na+/K+-ATPase alpha subunit, partial [Penaeus vannamei]	98.540	9.58E-95	298.0	5.25	4.70	-
DN2082_c0_g1	AFV69126.1 mitochondrial cytochrome c oxidase subunit Vb [Penaeus vannamei]	95.041	4.09E-86	248.0	5.24	4.42	0.83
DN6116_c0_g1	AOF79112.1 toll-3 [Penaeus japonicus]	46.429	0.006	32.3	3.53	5.22	-
DN10066_c0_g1	AKO62849.1 scavenger receptor B1 [Penaeus japonicus]	39.474	0.047	28.1	-	5.19	-
DN7387_c0_g1	ADR74382.1 prophenoloxidase-activating enzyme 2a [Penaeus monodon]	43.766	1.44E-98	298.0	5.17	2.91	-
DN7083_c0_g1	ADK66821.1 prophenoloxidase activating factor serine proteinase [Penaeus indicus]	56.522	3.20E-11	54.3	5.11	4.42	-
DN7243_c9_g1	AHV85235.1 ADAM metalloprotease, partial [Penaeus japonicus]	54.023	2.97E-52	176.0	2.12	-1.52	5.10
DN5387_c0_g1	AEG80154.1 Tcp-1-beta [Penaeus vannamei]	28.440	5.32E-16	77.0	5.07	4.71	-
DN7527_c5_g1	ADR31351.1 FKBP46 [Penaeus monodon]	44.068	5.63E-23	94.7	5.02	3.79	-1.45
DN7035_c0_g1	ALO17563.1 G-protein coupled receptor GRL101, partial [Penaeus monodon]	26.364	1.84E-04	40.0	5.02	3.52	-
DN6436_c0_g1	ADN43412.1 hemocyte homeostasis-associated protein [Penaeus monodon]	81.319	2.39E-53	164.0	4.95	4.00	-
DN8956_c0_g1	ADD63783.1 cytochrome P450 [Penaeus vannamei]	84.071	4.06E-62	194.0	2.43	-	4.92
DN7366_c9_g1	ADB65770.2 drosha [Penaeus japonicus]	46.154	0.043	32.0	4.38	4.91	-
DN7286_c0_g1	ADH94008.1 caspase [Penaeus japonicus]	78.646	4.18E-104	309.0	4.90	4.77	-
DN195_c0_g1	ALM25771.1 cyclin T [Penaeus monodon]	33.333	0.01	30.8	1.62	4.86	-
DN6389_c0_g1	AFQ62791.1 Bip [Penaeus vannamei]	97.308	1.88E-163	469.0	4.86	3.75	-

Table 2. Continued

	Result of blastx				Log2-transformed TMM-TPM value		
Trinity-transcripts	**Top hit of homology genes, gene name [spicies]**	**Identity (%)**	**E-Value**	**Bit-Score**	**Total**	**LEL^{Dim}**	**LEL^{Strong}**
DN7054_c0_g1	ARJ31757.1 casein kinase II subunit beta [Penaeus vannamei]	100	1.19E-141	401.0	4.85	4.83	-
DN7407_c4_g1	ADG22163.1 chitinase 3 precursor, partial [Penaeus monodon]	32.653	0.024	29.6	4.14	4.84	-
DN3088_c0_g1	BAM35674.1 putative reverse transcriptase [Penaeus japonicus]	78.571	8.66E-33	116.0	3.38	4.83	-
DN1031_c0_g1	AAK13497.1 syntenin, partial [Penaeus monodon]	100	5.12E-38	124.0	2.05	-	4.82
DN7016_c0_g1	ACN30234.1 ATP binding cassette transmembrane transporter [Penaeus vannamei]	25.405	2.22E-04	41.2	4.81	4.71	-
DN7071_c0_g1	AFE88208.1 proteasome alpha 3 [Penaeus monodon]	34.848	1.86E-19	82.4	4.79	4.11	-
DN1104_c0_g1	AEB00819.1 vasa-like protein [Penaeus japonicus]	60.000	0.036	28.5	3.70	4.78	-
DN7257_c0_g1	BAH86597.1 immune deficiency homolog [Penaeus japonicus]	99.375	1.96E-117	334.0	4.77	3.97	-
DN6934_c0_g2	ACU82846.1 acyl-CoA-binding protein [Penaeus chinensis]	39.773	4.74E-14	64.7	4.76	4.06	-
DN6261_c1_g1	AJE29369.1 vascular endothelial growth factor receptor precursor [Penaeus vannamei]	67.742	1.24E-22	88.2	4.71	4.74	-
DN3709_c0_g1	ABR01223.1 elongation factor 2 [Penaeus monodon]	44.697	5.05E-33	121.0	2.08	-0.56	4.73
DN6991_c0_g1	AJO69983.1 organellar Rab protein [Penaeus vannamei]	95.522	1.07E-37	137.0	4.66	4.61	-
DN4451_c0_g1	AXI69830.1 bystin 1 [Penaeus monodon]	92.000	8.32E-28	100.0	4.62	-	-
DN5402_c0_g1	ACC62172.1 innexin 2 [Penaeus monodon]	38.636	0.018	30.0	2.58	4.61	-
DN6775_c0_g1	AEI25987.1 hypothetical protein, partial [Penaeus monodon]	50.000	1.05E-05	40.4	4.61	3.22	-
DN7455_c0_g1	ACP19558.1 prophenoloxidase-activating enzyme [Penaeus monodon]	32.245	6.32E-26	106.0	4.60	3.91	-
DN7309_c0_g1	AAM73800.1 SCYLLA-like protein [Penaeus monodon]	43.182	2.24E-18	76.3	4.59	4.29	-
DN5317_c0_g2	AER34936.1 ubiquitin, partial [Penaeus vannamei]	31.818	2.84E-04	33.5	2.32	4.59	-
DN6969_c0_g1	AHI85755.1 cytoplasmic dynein intermediate chain [Penaeus chinensis]	97.222	1.20E-17	78.2	4.20	4.56	-
DN6926_c0_g1	ACU31809.1 alpha2 macroglobulin isoform 3, partial [Penaeus chinensis]	82.844	0	732.0	4.53	3.52	-
DN6618_c0_g1	AFE88208.1 proteasome alpha 3 [Penaeus monodon]	26.923	4.85E-07	47.4	4.51	3.91	-
DN5249_c0_g1	ACD13596.1 cyclophilin A [Penaeus monodon]	34.615	0.009	30.4	2.72	4.50	-
DN5366_c0_g1	ABV55648.1 Ubc protein, partial [Penaeus monodon]	33.333	1.37E-06	42.4	4.15	4.50	-
DN1688_c0_g1	AHX56189.1 activated protein kinase C receptor, partial [Penaeus vannamei]	33.333	5.23E-09	48.5	3.62	4.48	-
DN5675_c0_g1	AII17358.1 calmodulin, partial [Penaeus monodon]	33.333	0.001	35.8	4.47	3.20	-
DN10189_c0_g1	CAB85963.1 alpha glucosidase [Penaeus vannamei]	36.508	2.47E-05	38.5	2.26	4.45	-

Table 2. Continued

	Result of blastx				Log2-transformed TMM-TPM value		
Trinity-transcripts	**Top hit of homology genes, gene name [spicies]**	**Identity (%)**	**E-Value**	**Bit-Score**	**Total**	**LEL^{Dim}**	**LEL^{Strong}**
DN7420_c0_g2	AME17650.1 trypsin [Penaeus monodon]	33.992	2.72E-41	148.0	4.45	3.42	-
DN4635_c0_g1	AHV85235.1 ADAM metalloprotease, partial [Penaeus japonicus]	100	3.05E-31	115.0	4.45	3.43	-
DN6348_c0_g1	AIZ03630.1 cactin [Penaeus vannamei]	29.167	0.037	31.2	3.31	4.43	-
DN5487_c0_g1	ABI98679.1 ubiquitin-conjugating enzyme H5b [Penaeus vannamei]	100	4.17E-103	302.0	4.43	3.64	-
DN7339_c0_g1	AFW98991.1 prophenoloxidase activating enzyme [Penaeus vannamei]	39.615	1.29E-40	147.0	4.42	2.70	-
DN6516_c0_g1	AFE88208.1 proteasome alpha 3 [Penaeus monodon]	36.364	2.36E-14	67.8	4.42	3.61	-
DN5578_c0_g1	AWI47734.1 DEAD box-ATP dependent RNA helicase 48 [Penaeus monodon]	32.632	2.74E-33	125.0	4.38	2.40	-
DN5803_c0_g1	AHK23065.1 double-stranded RNA-specific adenosine deaminase [Penaeus japonicus]	28.169	1.36E-06	47.4	4.37	4.22	-
DN6155_c0_g2	ACO59906.1 eukaryotic translation initiation factor 3 subunit G [Penaeus monodon]	28.571	7.81E-04	36.6	3.60	-1.78	4.34
DN7161_c0_g1	ACR15870.1 serine protease [Penaeus chinensis]	45.763	1.89E-62	201.0	4.34	3.05	-
DN7465_c4_g1	ANZ80593.1 ubiquitin-activating enzyme E1 [Penaeus monodon]	27.000	0.03	33.1	4.34	2.84	-
DN1554_c0_g1	ADQ43366.1 HMGBa [Penaeus vannamei]	97.826	6.62E-60	185.0	3.32	-	4.30
DN7101_c0_g1	ADR74382.1 prophenoloxidase-activating enzyme 2a [Penaeus monodon]	29.218	3.50E-24	101.0	4.30	4.23	-
DN10270_c0_g1	CAB85963.1 alpha glucosidase [Penaeus vannamei]	50.000	1.94E-05	37.7	4.28	2.04	-
DN5426_c0_g1	AJE29369.1 vascular endothelial growth factor receptor precursor [Penaeus vannamei]	60.317	9.05E-25	93.2	3.97	4.28	-
DN6353_c0_g1	BAE78496.1 farnesoic acid O-methyltransferase [Penaeus japonicus]	100	6.11E-65	208.0	3.74	4.28	-
DN6712_c0_g1	AGZ01980.1 valosin-containing protein [Penaeus monodon]	39.264	1.17E-27	109.0	4.26	3.68	-
DN1895_c0_g1	AWB51937.1 60S ribosomal protein L7A [Penaeus vannamei]	65.625	1.41E-40	132.0	3.06	4.26	-
DN7362_c0_g2	ABW88999.1 double WAP domain-containing protein [Penaeus japonicus]	38.636	0.018	29.6	1.82	4.26	-
DN6515_c0_g1	AON76443.1 defender against apoptotic death [Penaeus japonicus]	100	7.21E-80	231.0	4.25	4.03	-
DN7393_c11_g4	AHH32888.1 integrin alpha 5 [Penaeus chinensis]	84.058	5.20E-33	116.0	4.24	4.16	-
DN9011_c0_g1	AEU11366.1 Broad-complex protein isoform 4 [Penaeus monodon]	45.455	1.15E-04	35.8	1.83	4.24	-
DN10350_c0_g1	AHF21001.1 activated C kinase 1 receptor [Penaeus japonicus]	48.276	0.01	30.0	0.98	4.23	-
DN3546_c0_g1	BAJ23879.1 glycogen phosphorylase [Penaeus japonicus]	100	3.56E-105	325.0	4.21	2.98	-
DN7001_c1_g1	ATY51983.1 cAMP-responsive element binding protein-3, partial [Penaeus vannamei]	97.170	7.85E-139	421.0	4.19	3.70	-
DN7481_c12_g1	ADO32581.1 cathepsin B [Penaeus japonicus]	99.699	0	675.0	4.17	3.82	-

Table 2. Continued

	Result of blastx				Log2-transformed TMM-TPM value		
Trinity-transcripts	**Top hit of homology genes, gene name [spicies]**	**Identity (%)**	**E-Value**	**Bit-Score**	**Total**	**LEL^{Dim}**	**LEL^{Strong}**
DN5852_c0_g1	ABW88999.1 double WAP domain-containing protein [Penaeus japonicus]	55.172	5.13E-05	36.2	3.65	4.16	-
DN8034_c0_g1	sp|Q9NGP0.1 Crustacean hyperglycemic hormone B	32.609	0.005	30.8	2.56	4.16	-
DN5575_c0_g1	AOZ86849.1 tyrosine-protein phosphatase non-receptor type 2 [Penaeus japonicus]	100	1.12E-77	235.0	3.41	4.16	-
DN4_c0_g1	ADO00929.1 endoplasmic reticulum protein 57 [Penaeus monodon]	98.182	0	544.0	4.15	4.07	-
DN7346_c7_g2	sp|Q25456.1 Allergen Met e I	72.139	8.55E-60	193.0	4.15	2.23	-
DN7367_c0_g1	ABO33174.1 serine protease-like protein 3 [Penaeus monodon]	90.612	6.69E-166	469.0	4.13	3.25	-
DN7272_c0_g1	ACR23314.1 chitinase 4 precursor [Penaeus vannamei]	39.024	0.008	36.6	4.13	3.40	-
DN2737_c0_g2	AEC12821.1 lactate dehydrogenase [Penaeus vannamei]	34.921	0.025	33.1	3.54	4.12	-
DN1723_c0_g1	AYF59253.1 tyrosine-protein phosphatase non-receptor type 2 [Penaeus monodon]	37.313	1.47E-11	55.1	2.64	4.12	-
DN6691_c0_g1	AEX07320.1 gamma-interferon-inducible lysosomal thiol reductase [Penaeus merguiensis]	88.614	1.87E-139	387.0	4.12	3.22	-
DN7378_c8_g1	AHH32891.1 integrin beta 6, partial [Penaeus chinensis]	76.176	1.28E-102	309.0	4.12	3.45	-
DN5417_c0_g1	ACD13598.1 variant transformer-2 protein [Penaeus monodon]	36.986	3.48E-06	45.1	4.12	3.84	-
DN6751_c1_g1	AFW98986.1 prophenoloxidase activating factor [Penaeus chinensis]	50.735	2.16E-93	281.0	4.11	0.96	-
DN7290_c1_g4	AEU11365.1 Broad-complex protein isoform 6 variant 2 [Penaeus monodon]	42.254	1.17E-19	78.2	3.77	4.09	-
DN3814_c0_g1	AEU11365.1 Broad-complex protein isoform 6 variant 2 [Penaeus monodon]	51.515	3.68E-07	42.7	2.67	4.09	-
DN4230_c0_g1	AQW41373.1 peroxiredoxin [Penaeus monodon]	96.386	2.95E-55	168.0	2.09	4.07	-
DN9388_c0_g1	BAM37459.1 argonaute 2 [Penaeus japonicus]	100	1.89E-51	169.0	4.06	-	-
DN7527_c1_g1	sp|Q6PV61.3|H2A PENVA RecName: Full=Histone H2A	61.765	4.60E-35	117.0	3.99	-1.78	-
DN1562_c0_g2	ADC55251.1 mitochondrial ATP synthase subunit alpha precursor [Penaeus vannamei]	98.462	1.17E-36	126.0	3.89	-	-
DN6093_c0_g1	BBD20111.1 wsv343-like protein [Penaeus japonicus]	33.333	0.004	31.6	3.75	-	-
DN7367_c1_g1	ABO33174.1 serine protease-like protein 3 [Penaeus monodon]	89.474	1.52E-15	66.2	3.69	-	-
DN6384_c0_g1	ACJ36226.1 spermatogonial stem-cell renewal factor [Penaeus chinensis]	93.827	3.90E-47	152.0	3.62	-	-
DN7906_c0_g1	ABC33917.1 trypsin-like serine proteinase [Penaeus chinensis]	48.980	9.85E-13	58.2	3.62	-	-
DN6432_c0_g2	ACM91676.1 F1F0-ATP synthase beta subunit [Penaeus japonicus]	34.524	1.59E-05	38.9	3.60	-	-
DN4683_c0_g1	AMQ76360.1 clathrin coat assembly protein 17 [Penaeus vannamei]	96.429	4.39E-35	113.0	3.58	-	-
DN2353_c0_g1	AEG80154.1 Tcp-1-beta [Penaeus vannamei]	38.571	5.01E-08	45.1	3.56	-	-

Table 2. Continued

	Result of blastx				Log2-transformed TMM-TPM value		
Trinity-transcripts	**Top hit of homology genes, gene name [spicies]**	**Identity (%)**	**E-Value**	**Bit-Score**	**Total**	**LEL^{Dim}**	**LEL^{Strong}**
DN8431_c0_g1	AEU11366.1 Broad-complex protein isoform 4 [Penaeus monodon]	55.263	9.42E-11	53.1	3.46	-	-
DN10199_c0_g1	AGS38337.1 ERK [Penaeus vannamei]	100	7.87E-11	52.8	3.45	-	-
DN1727_c0_g1	AGN53342.1 peroxinectin [Penaeus indicus]	53.704	1.40E-05	39.3	3.43	-	-
DN6840_c0_g3	ADK94870.1 saposin isoform 1 [Penaeus monodon]	95.522	8.14E-40	135.0	3.42	-	-
DN7505_c0_g1	AIU96362.1 peroxinectin [Penaeus merguiensis]	90.654	2.49E-63	202.0	3.42	-	-
DN5384_c0_g3	ARE29775.1 p53-like protein isoform delta [Penaeus vannamei]	91.549	1.37E-40	132.0	3.40	-	-
DN1308_c0_g2	AMQ76360.1 clathrin coat assembly protein 17 [Penaeus vannamei]	54.098	1.00E-18	72.0	3.38	-	-
DN7379_c0_g5	AHA90856.1 FEM-1 [Penaeus vannamei]	38.333	6.57E-06	38.9	3.38	-	-
DN9787_c0_g1	ADV04044.1 ATP-binding cassette transmembrane transporter [Penaeus chinensis]	42.857	0.01	30.0	3.33	-	-
DN1403_c0_g1	AEU11366.1 Broad-complex protein isoform 4 [Penaeus monodon]	54.839	1.07E-06	42.4	3.32	-	-
DN9524_c0_g1	AMK05616.1 transforming growth factor beta regulator 1-like protein [Penaeus monodon]	100	1.93E-52	163.0	3.32	-	-
DN529_c0_g1	AEE25939.1 V-H-ATPase subunit A [Penaeus vannamei]	93.151	5.34E-44	147.0	3.32	-	-
DN6000_c0_g2	AGU41814.1 IRAK1 protein [Penaeus vannamei]	51.852	0.008	30.4	3.32	-	-
DN5252_c0_g1	AKV16260.1 adiponectin receptor [Penaeus vannamei]	43.243	0.038	29.3	3.29	-	-
DN31_c0_g1	AFJ59949.1 M-type lectin [Penaeus japonicus]	52.542	3.72E-13	60.8	3.28	-	-
DN7393_c11_g2	AHH32888.1 integrin alpha 5 [Penaeus chinensis]	68.047	3.59E-73	235	3.27	-	-
DN8798_c0_g1	AHX84170.1 target of rapamycin [Penaeus chinensis]	41.538	1.51E-07	45.1	3.26	-	-
DN1960_c0_g1	AEB96259.1 C-type lectin [Penaeus merguiensis]	39.286	0.022	28.9	3.22	-	-
DN8546_c0_g1	AGG82488.1 p38 [Penaeus vannamei]	39.286	2.28E-04	34.7	3.15	-	-
DN4511_c0_g1	ARO77487.1 MST4 [Penaeus vannamei]	34.328	2.12E-07	43.9	3.14	-	-
DN773_c0_g1	ACO59906.1 eukaryotic translation initiation factor 3 subunit G [Penaeus monodon]	32.787	0.021	29.6	3.11	-	2.13
DN8565_c0_g1	AOF79107.1 dorsal [Penaeus japonicus]	100	2.20E-30	108.0	3.10	-	-
DN3666_c0_g1	AEU11364.1 Broad-complex protein isoform 6 variant 1 [Penaeus monodon]	44.118	2.31E-17	72.0	3.09	-	-
DN3692_c0_g1	AFX71574.1 dopamine receptor type 1 [Penaeus monodon]	28.788	1.15E-04	37.4	3.08	-	-
DN9385_c0_g1	AAK28535.1 farnesoic acid O-methyltransferase [Metapenaeus ensis]	33.333	0.046	28.1	3.08	-	-
DN8172_c0_g1	AEU11366.1 Broad-complex protein isoform 4 [Penaeus monodon]	46.429	4.71E-04	34.3	3.06	-	-

Table 2. Continued

	Result of blastx				Log2-transformed TMM-TPM value		
Trinity-transcripts	**Top hit of homology genes, gene name [spicies]**	**Identity (%)**	**E-Value**	**Bit-Score**	**Total**	**LEL^{Dim}**	**LEL^{Strong}**
DN10540_c0_g1	AFK25798.1 KPI2 [Penaeus vannamei]	28.814	0.042	28.1	3.05	-	-
DN6873_c0_g2	ADH93870.1 caspase [Penaeus japonicus]	29.688	0.006	32.0	3.04	-	-
DN8186_c0_g1	BAO01182.1 protein phosphatase 1B [Penaeus japonicus]	100	2.49E-48	154.0	3.03	-	-
DN8634_c0_g1	BAH57291.1 putative ovarian lipoprotein receptor [Penaeus japonicus]	35.294	0.014	30.0	3.03	-	-
DN8031_c0_g1	AWI47734.1 DEAD box-ATP dependent RNA helicase 48 [Penaeus monodon]	47.222	4.19E-07	42.4	3.03	-	-
DN8047_c0_g1	AEU11366.1 Broad-complex protein isoform 4 [Penaeus monodon]	65.714	5.10E-09	47.8	3.03	-	-
DN1775_c0_g1	AFW98985.1 prophenoloxidase activating enzyme [Penaeus chinensis]	40.367	4.19E-24	93.2	3.02	-	-

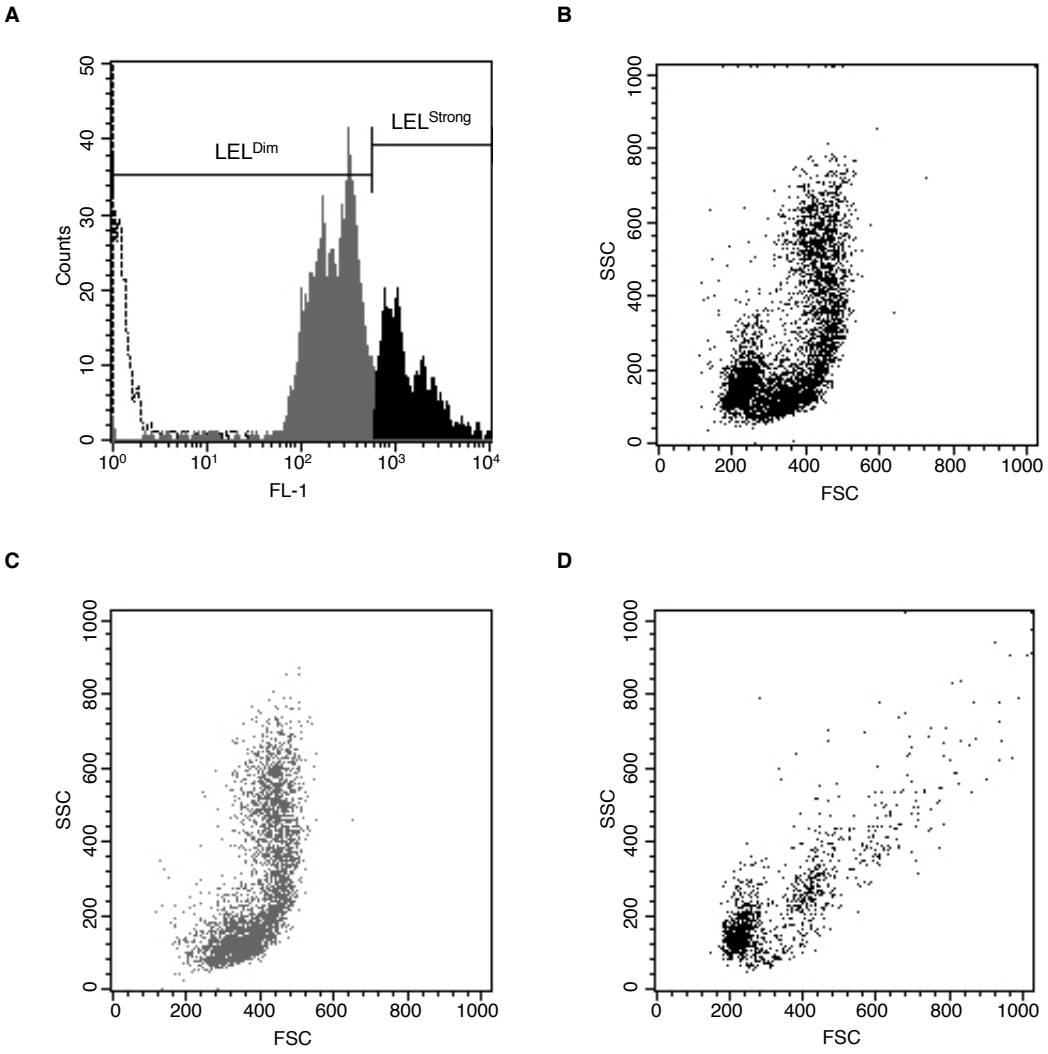


Figure 1. Flow cytometry analysis of LEL-stained hemocytes from a shrimp. The intensity of FL-1 signal of LEL-stained hemocytes (A). Dotted line indicates negative control of FL-1 value. Dot-plot analysis of total hemocytes (B) LEL^{Dim} hemocytes (C) and LEL^{Strong} hemocytes (D). X- and Y-axes indicate FSC and SSC, respectively.

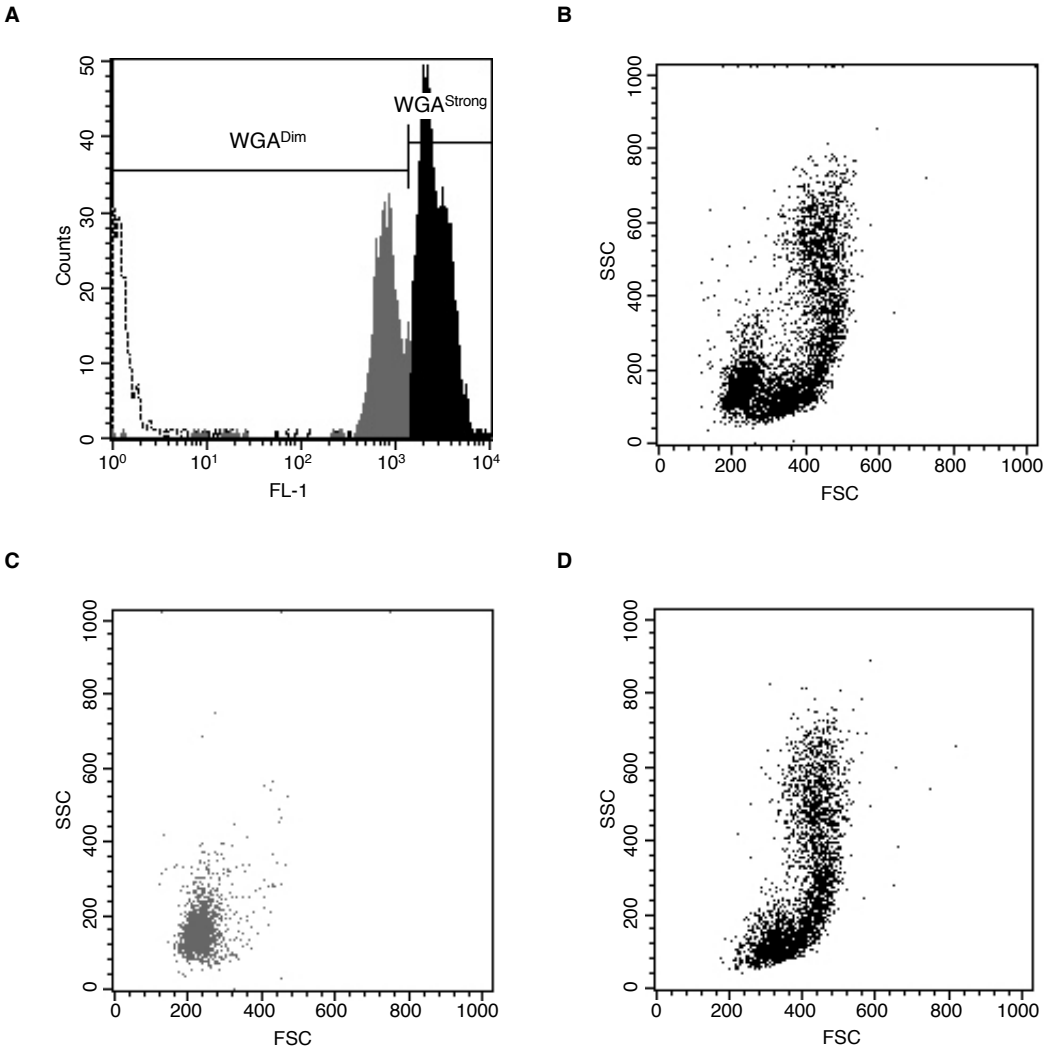


Figure 2. Flow cytometry analysis of WGA-stained hemocytes from a shrimp. The intensity of FL-1 signal of WGA-stained hemocytes (A). Dotted line indicates negative control of FL-1 value. Dot-plot analysis of total hemocytes (B) WGA^{Dim} hemocytes (C) and WGA^{Strong} hemocytes (D). X- and Y-axes indicate FSC and SSC, respectively.

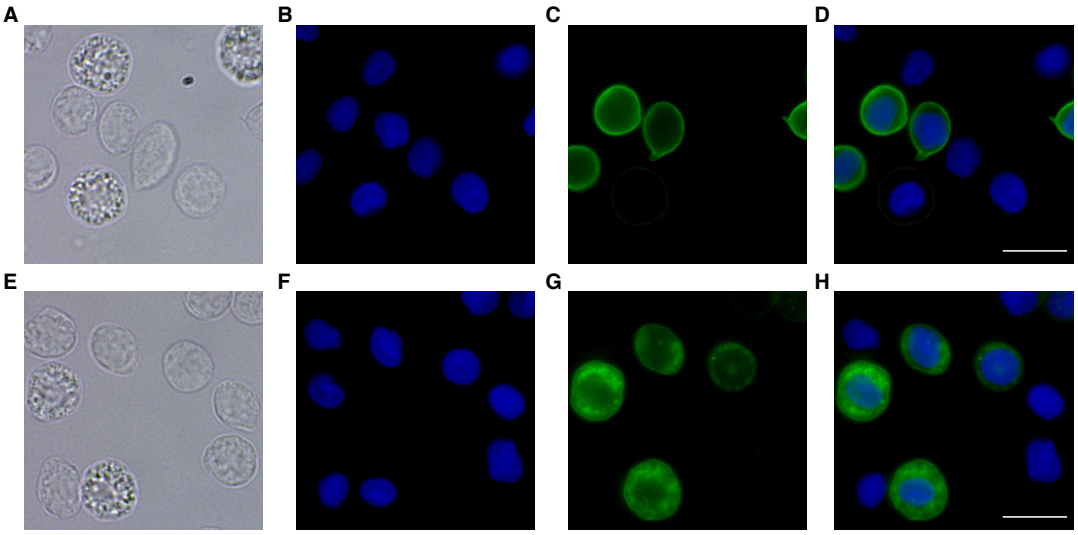


Figure 3. Lectin staining of total hemocytes from a shrimp. Hemocytes stained LEL (A-D) and WGA (E-H). Bright-field (A, E). Nucleolus stained as blue by Hoechst 33258 (B, F). Each fluorescent lectin stained as green, LEL (C) and WGA (G). Merged figure (D, H). Bars indicate 10 μm scale.

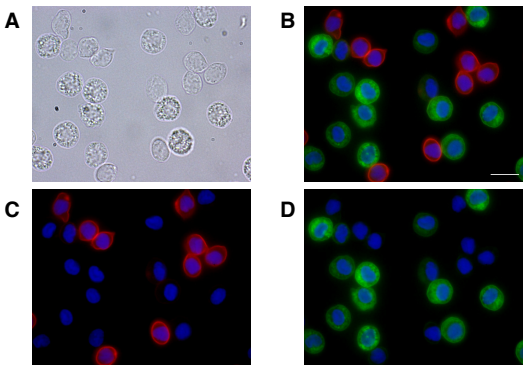


Figure 4. Double lectin staining of total hemocytes from a shrimp. Hemocytes stained LEL and WGA. Bright-field (A). Nucleolus stained by Hoechst 33258 as blue, hemocytes stained by LEL as red and WGA as green (B). Nucleolus stained by Hoechst 33258 as blue, hemocytes stained by LEL as red (C). Nucleolus stained by Hoechst 33258 as blue, hemocytes stained by WGA as green (D). Bars indicate 10 μm scale.

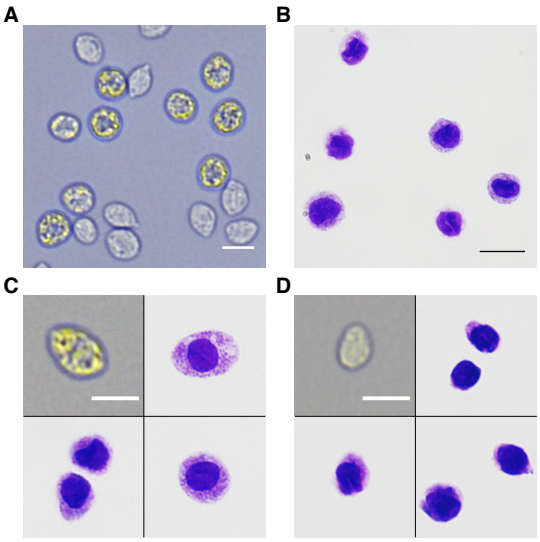


Figure 5. Bright field microscopic observation and May-Giemsa staining of hemocytes from a shrimp. Total hemocyte observed under bright-field (A). Total hemocytes stained by May-Giemsa staining (B). Bright field observation and May-Giemsa staining of LEL^{Dim} hemocytes (C) and LEL^{Strong} hemocytes (D). Bars indicate 10 μm scale.

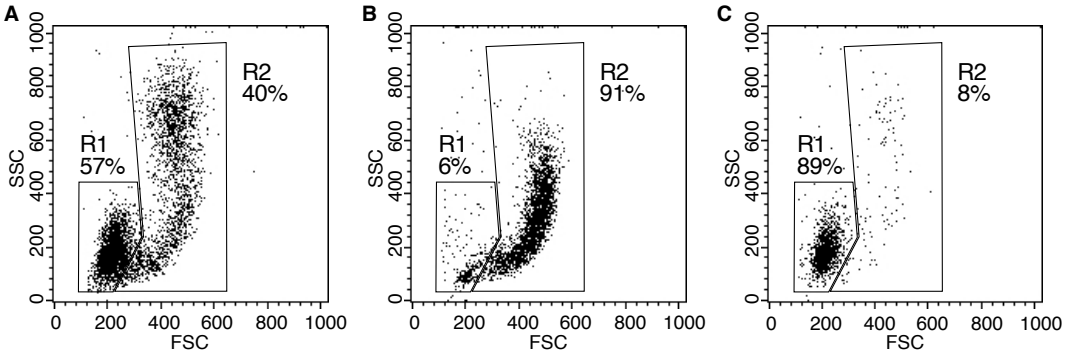
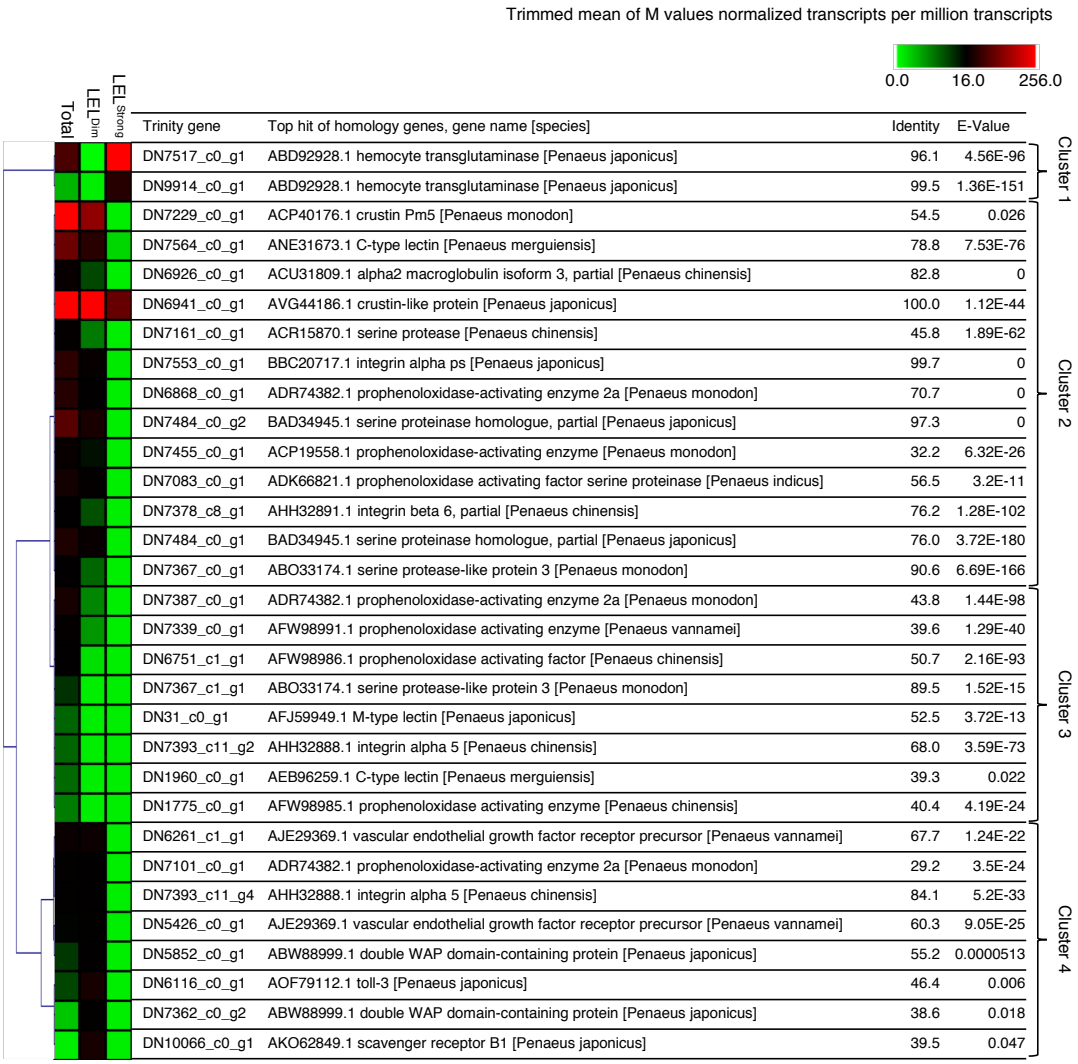


Figure 6. Dot plot analyses of total, LEL^{Dim} and LEL^{Strong} hemocytes from a shrimp. Total hemocytes (A), LEL^{Dim} hemocytes (B) and LEL^{Strong} hemocytes (C). Each region was established based on characteristic cell plots. X- and Y-axes indicate FSC and SSC, respectively.



Trinity gene	Top hit of homology genes, gene name [species]	Identity	E-Value	
DN7517_c0_g1	ABD92928.1 hemocyte transglutaminase [Penaeus japonicus]	96.1	4.56E-96	Cluster 1
DN9914_c0_g1	ABD92928.1 hemocyte transglutaminase [Penaeus japonicus]	99.5	1.36E-151	
DN7229_c0_g1	ACP40176.1 crustin Pm5 [Penaeus monodon]	54.5	0.026	Cluster 2
DN7564_c0_g1	ANE31673.1 C-type lectin [Penaeus merguiensis]	78.8	7.53E-76	
DN6926_c0_g1	ACU31809.1 alpha2 macroglobulin isoform 3, partial [Penaeus chinensis]	82.8	0	
DN6941_c0_g1	AVG44186.1 crustin-like protein [Penaeus japonicus]	100.0	1.12E-44	
DN7161_c0_g1	ACR15870.1 serine protease [Penaeus chinensis]	45.8	1.89E-62	
DN7553_c0_g1	BBC20717.1 integrin alpha ps [Penaeus japonicus]	99.7	0	
DN6868_c0_g1	ADR74382.1 prophenoloxidase-activating enzyme 2a [Penaeus monodon]	70.7	0	
DN7484_c0_g2	BAD34945.1 serine proteinase homologue, partial [Penaeus japonicus]	97.3	0	
DN7455_c0_g1	ACP19558.1 prophenoloxidase-activating enzyme [Penaeus monodon]	32.2	6.32E-26	
DN7083_c0_g1	ADK66821.1 prophenoloxidase activating factor serine proteinase [Penaeus indicus]	56.5	3.2E-11	
DN7378_c8_g1	AHH32891.1 integrin beta 6, partial [Penaeus chinensis]	76.2	1.28E-102	
DN7484_c0_g1	BAD34945.1 serine proteinase homologue, partial [Penaeus japonicus]	76.0	3.72E-180	
DN7367_c0_g1	ABO33174.1 serine protease-like protein 3 [Penaeus monodon]	90.6	6.69E-166	
DN7387_c0_g1	ADR74382.1 prophenoloxidase-activating enzyme 2a [Penaeus monodon]	43.8	1.44E-98	Cluster 3
DN7339_c0_g1	AFW98991.1 prophenoloxidase activating enzyme [Penaeus vannamei]	39.6	1.29E-40	
DN6751_c1_g1	AFW98986.1 prophenoloxidase activating factor [Penaeus chinensis]	50.7	2.16E-93	
DN7367_c1_g1	ABO33174.1 serine protease-like protein 3 [Penaeus monodon]	89.5	1.52E-15	
DN31_c0_g1	AFJ59949.1 M-type lectin [Penaeus japonicus]	52.5	3.72E-13	
DN7393_c11_g2	AHH32888.1 integrin alpha 5 [Penaeus chinensis]	68.0	3.59E-73	
DN1960_c0_g1	AEB96259.1 C-type lectin [Penaeus merguiensis]	39.3	0.022	
DN1775_c0_g1	AFW98985.1 prophenoloxidase activating enzyme [Penaeus chinensis]	40.4	4.19E-24	
DN6261_c1_g1	AJE29369.1 vascular endothelial growth factor receptor precursor [Penaeus vannamei]	67.7	1.24E-22	Cluster 4
DN7101_c0_g1	ADR74382.1 prophenoloxidase-activating enzyme 2a [Penaeus monodon]	29.2	3.5E-24	
DN7393_c11_g4	AHH32888.1 integrin alpha 5 [Penaeus chinensis]	84.1	5.2E-33	
DN5426_c0_g1	AJE29369.1 vascular endothelial growth factor receptor precursor [Penaeus vannamei]	60.3	9.05E-25	
DN5852_c0_g1	ABW88999.1 double WAP domain-containing protein [Penaeus japonicus]	55.2	0.0000513	
DN6116_c0_g1	AOF79112.1 toll-3 [Penaeus japonicus]	46.4	0.006	
DN7362_c0_g2	ABW88999.1 double WAP domain-containing protein [Penaeus japonicus]	38.6	0.018	
DN10066_c0_g1	AKO62849.1 scavenger receptor B1 [Penaeus japonicus]	39.5	0.047	

Figure 7. Hierarchical clustering analysis of immune-related trinity-transcripts extracted as differentially expressed in total, LEL^{Dim} and LEL^{Strong} hemocytes. Each column is the TMM-TPM value. Relatively highly expressed trinity-transcripts are shown in red, relatively weakly expressed trinity-transcripts are shown in green.

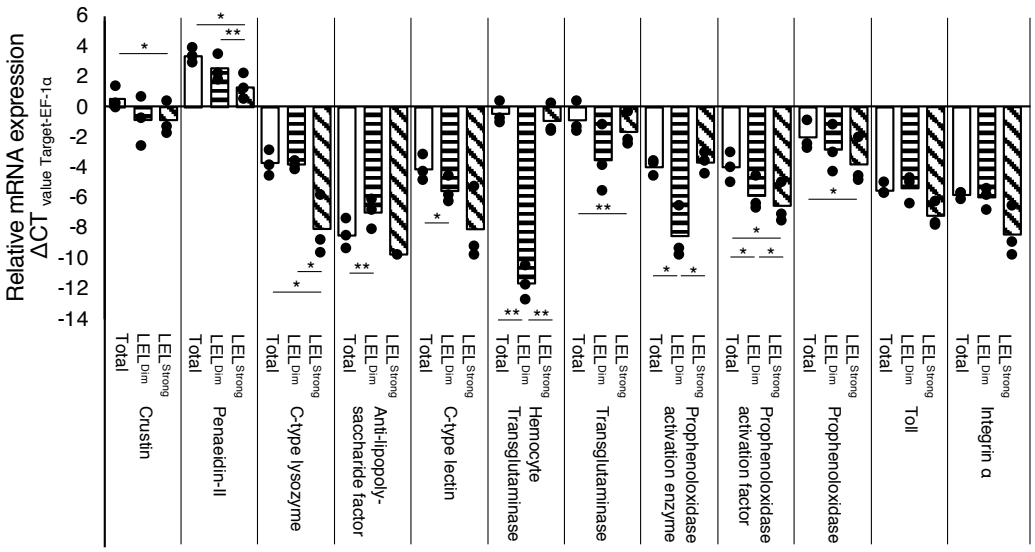


Figure 8. qRT-PCR analyses of 12 transcripts. ΔCt values analyzed by qRT-PCR. Higher ΔCT value indicates higher expression. Each bar indicates the average value. Double asterisk (**) on the bars indicates p-value less than 0.01 and single asterisk (*) indicates p-value less than 0.05.

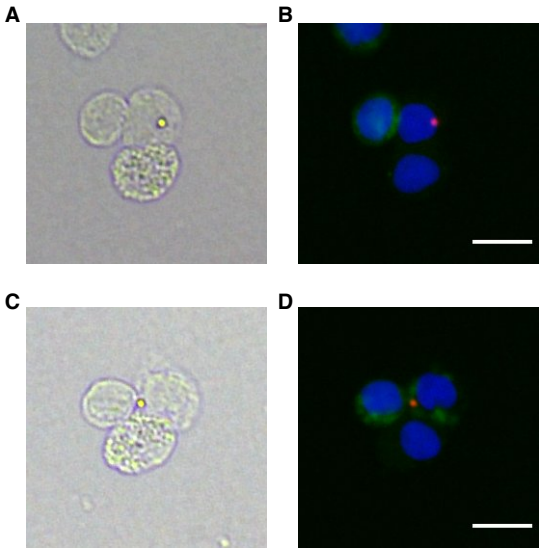


Figure 9. LEL staining on hemocytes phagocyted micro beads. Microscopic observation under bright-field (A, C) and under fluorescent-field (B, D). Nucleolus stained by Hoechst 33258 as blue, hemocytes stained by LEL as green and phagocytized beads as red (B, D). Bars indicate 10 μm scale.

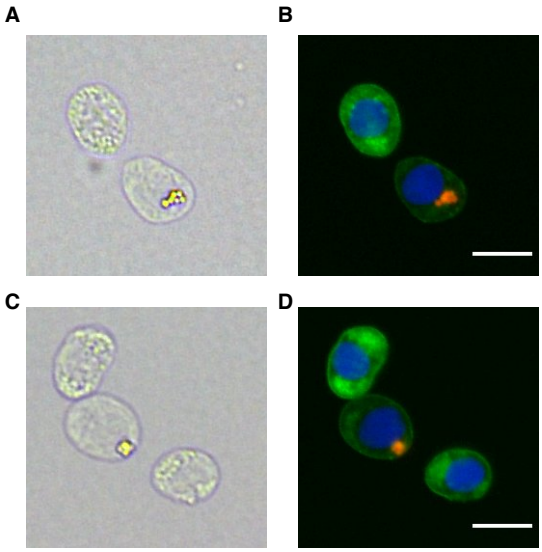


Figure 10. WGA staining on hemocytes phagocyted micro beads. Microscopic observation under bright-field (A, C) and under fluorescent-field (B, D). Nucleolus stained by Hoechst 33258 as blue, hemocytes stained by WGA as green and phagocytized beads as red (B, D). Bars indicate 10 μm scale.

第５章　総括

クルマエビ類を含む甲殻類血球細胞の研究は未だ発展途上である．第 2 章では，組織中に定着している血球細胞機能の分子生物学的機能解析を，次世代シークエンサーによる網羅的転写産物解析により行った．その結果，心臓およびリンパ様器官中の血球細胞は循環している血球細胞とは蓄積する転写産物が明らかに異なり，機能も異なることが推察された．一方，エラの血球細胞は循環血球細胞と類似した免疫関連遺伝子の転写産物蓄積パターンを見せた．これは他の免疫関連臓器と比較して，エラが異物に遭遇しやすい環境のためだと考えられた．これまで組織中の血球細胞は固着性血球細胞と称され，循環血球細胞と異なる細胞群であるという見方もあったが，フローサイトメーター解析のプロット図からは両者に明確な差が見られなかった．固着性血球細胞および血球細胞間を明確に分類するマーカーもしくは手法は未だ報告がないため，本研究では組織中の血球細胞は循環血球細胞の一部であると結論づけた．

第 3 章では，貪食能を有する血球細胞 (貪食血球細胞) の特異的細胞表面分子を探索するため磁気ビーズを用いた貪食血球細胞の濃縮手法を開発した．さらに，本手法により濃縮された貪食血球細胞で特異的に蓄積される遺伝子転写産物を同定し，抗体を用いた免疫学的染色により本遺伝子が貪食血球細胞のマーカーとなり得るかを評価した．磁気ビーズを用いる手法は簡便であり高価な機器を必要としないため，甲殻類の貪食血球細胞研究において有用なツールとなる可能性が示唆された．次世代シークエンサーによる網羅的な遺伝子転写産物解析の結果，ビーズを貪食した血球細胞ではインテグリン遺伝子の転写産物の蓄積が多いことが判明した．本インテグリンに対するポリクローナル抗体を作製し，免疫学的染色を行った結果，全

血球細胞はインテグリン陽性および陰性細胞の 2 種類に分類されたのに加え，ビーズ貪食血球細胞にもインテグリン陽性および陰性細胞が存在することが明らかとなった．本結果より，クルマエビの貪食血球細胞には少なくとも 2 種類の集団が存在することが示唆された．また，昆虫類ではインテグリンが顆粒球のマーカー遺伝子として報告があることから，本インテグリン抗体もクルマエビの顆粒球を染色したことが予想された．

第 4 章では，血球細胞を糖鎖に基づき分類するため，レクチンの血球細胞に対する反応性をフローサイトメーターおよび蛍光顕微鏡観察により確認した．その結果，クルマエビの血球細胞には，LEL および WGA が特徴的な染色性を示した．LEL は細胞表面に強い結合性を示す一方，WGA は細胞内の顆粒に強い結合を示すことが蛍光顕微鏡下で観察された．さらに，ビオチン標識された LEL およびストレプトアビジン標識マイクロビーズを用いた細胞磁気標識法により，2 つの血球細胞亜集団の分離に成功した．これら細胞集団は形態学的特徴およびその遺伝子転写産物の蓄積パターンから，既報の無顆粒球および顆粒球の 2 種類の集団であることが示唆された．また，ビーズを貪食した血球細胞に対して WGA が 100%の陽性率を示したことから，クルマエビにおいて顆粒球が貪食作用を主に担っていることが示唆された．

本研究から得られた結果より，クルマエビ血球細胞の分類に抗体やレクチンを用いることが有効であることが判明した．しかしながら，課題も多く残る．第一に，第 2 章および第 4 章のフローサイトメーター解析のプロット図が異なるように，実験に用いる抗凝固液や固定液の影響で血球細胞の形態が変化する現象が見受けられた．諸言でも述べた通り，クルマエビ類の血球細胞は未固定の状態では物理的

な刺激に弱く，また，サンプリング時に用いる抗凝固液または固定液の種類によってその形態を変化させる．さらに現在，各国の研究室間では異なるサンプリング手法，抗凝固液および固定液が用いられており，血球細胞分類の比較を異なる研究室間で行うことは非常に困難である．そのため，最適な抗凝固液，固定方法および染色方法の開発および標準化は今後のクルマエビ類免疫研究において重要な課題である．第 4 章において血球細胞の分取に成功したが，本血球細胞はホルマリンにより固定されていた．ホルマリンによる固定は核酸の抽出を困難にするため，核酸抽出に影響を及ぼさない分類および固定方法の検討も今後の血球細胞研究には必要である．これらの課題を解決し，これまで報告されてきたマーカー遺伝子や糖鎖の組成，形態学的特徴，遺伝子転写産物および細胞機能を複合的に研究することがクルマエビ類の免疫機構および造血機構の解明に繋がる．

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