

Characterization of Shrimp White Spot Syndrome Virus (WSSV) Structural Protein VP51A (ORF294)

周宗錄、張雲祥

E-mail: 9607789@mail.dyu.edu.tw

ABSTRACT

White spot syndrome virus (WSSV) is an important crustacean virus causing high mortality in cultured shrimp. WSSV is a double-stranded DNA virus with a genome size of about 300 kbp. So far, 58 viral structural proteins were identified. In this research, one of the structural protein translated form ORF294 (GeneBank accession no. AF440570), the VP51A, was studied. Gene structure analysis showed that the transcription initiation site of vp51A was located 135 bp upstream of the translation start codon ATG. TATA box, or its related consensus sequence was not recognized is 5' untranslated region of this gene. The poly-A addition signal was overlapped with the translation stop codon TAA and the poly-A tail was added 23 bp downstream of the stop codon. The vp51A transcripts was observed 6 hours after virus infection and the expression levels increasing with the infection time course. Computer software anlysis discovered a conserved sequence of the nuclear localization signal (NLS) between 37 and 43 of VP51A coding region, but such prediction wasn't confirmed by the following in vitro analysis performed in Sf9 cells. Immunoelectron microscopy analysis and Western blot hybridization performed on intact virus particle and separated viral components showed that the VP51A is an envelope protein. Furthermore, Western blot analysis of WSSV virion also demonstrated that except the expected 53 kDa band, there were another protein bands such as an obvious signal around 72 kDa and some other small molecular weight proteins exist. Similar result was found in the Western blot results performed on WSSV infect shrimp tissues and recombinant VP51A expressed insect cells. But when using the in vitro transcription and translation system to express the recombinant VP51A it demonstrated a 72 kDa protein only. This result showed that the VP51A gene might expresses a large molecular weight protein first and it will then be processed into another lower molecular weight ones. Other experiments, including the predict protein cutting site mutation of VP51A and Western blot hybridization by VP51A different region fragments derived antibodies suggested that most of the cutting sites of VP51A might distribute closer to the N-terminal region. The is processed and what are the biological meanings of there different types of VP51A proteins, still left to be elocudate.

Keywords : WSSV ; structural protein ; envelope protein

Table of Contents

封面內頁 簽名頁 授權書.....	iii	中文摘要.....	iv	英文摘要.....	vi
謝.....	viii	目錄.....	x	圖目錄.....	xiii
錄.....	xv	1. 前言.....	1	1.1 白點症病毒.....	1
性.....	5	1.3 白點症病毒結構性蛋白質之發現與應用.....	5	1.4 研究目的.....	7
法.....	9	2.1 基因結構分析.....	9	2.1.1 樣品來源.....	9
取.....	9	2.1.3 反轉錄聚合?鏈反應(reverse transcription polymerase chain reaction, RT-PCR).....	10	2.1.4 基因表現時序分析.....	11
序分析.....	11	2.1.5 快速擴增cDNA 5' / 3' 端.....	11	2.2 蛋白質表現及特性分析.....	14
之細胞內表現位置分析.....	14	2.2.2 抗體製備.....	16	2.2.3 VP51A於病毒顆粒之定位分析.....	18
2.2.3.1 白點症病毒純化.....	18	2.2.3.2 白點症病毒之抗VP51A C端抗體西方轉漬分析.....	19	2.2.3.3 以西方轉漬分析VP51A於白點症病毒顆粒上之定位.....	20
2.2.3.4 以免疫電顯分析VP51A於白點症病毒顆粒上之定位.....	20	2.2.4 VP51A分子量變異之分析.....	21	2.2.4.1 VP51A試管內轉錄及轉譯 (Transcription/ Translation Systems, TNT)分析.....	21
2.2.4.2 昆蟲細胞Sf9表現重組VP51A之抗V5抗體西方轉漬分析.....	22	2.2.4.3 白點症病毒顆粒、病毒感染蝦組織及昆蟲細胞Sf9抗VP51A C端抗體西方轉漬分析... 23	23	2.2.4.4 白點症病毒顆粒、病毒感染蝦組織及昆蟲細胞Sf9抗VP51A中段片段抗體西方轉漬分析	23
2.2.4.5 VP51A切割位分析.....	24	3. 結果與討論.....	25	3.1 基因結構分析.....	25
5' / 3' 端.....	26	3.2 蛋白質表現及特性分析.....	26	3.2.1 VP51A之細胞內表現位置分析.....	26
VP51A於病毒顆粒之定位分析.....	27	3.2.3 VP51A分子量變異之分析.....	28	3.2.3.1 VP51A試管內轉錄及轉譯分析.....	28
28	28	3.2.3.2 昆蟲細胞Sf9表現重組VP51A之抗V5抗體西方轉漬分析.....	29	3.2.3.3 白點症病毒顆粒、病毒感染蝦組織及昆蟲細胞Sf9抗VP51A C端抗體西方轉漬分析.....	29
29	29	3.2.3.4 白點症病毒顆粒、病毒感染蝦組織及昆蟲細胞Sf9抗VP51A中段片段抗體西方轉漬分析.....	30	3.2.3.5 VP51A切割位分析.....	30
30	30	4. 結論.....	31	參	31

REFERENCES

1. 王涵青, 2002。蝦白點症病毒四種基因轉錄分析之研究。國立台灣大學海洋研究所碩士論文。
2. Bartosch, B., Vitelli, A., and Granier, C. (2003) Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J Biol Chem* 278:41624-41630.
3. Bartenschlager, R., and Lohmann, V. (2000) Replication of hepatitis C virus. *J Gen Virol* 81:1631-1648.
4. Bielecki, L., Hindley, C., and Talbot, S. J. (2004) A polypyrimidine tract facilitates the expression of Kaposi's sarcoma-associated herpesvirus vFLIP through an internal ribosome entry site. *J Gen Virol* 85:615-620.
5. Bielecki, L., and Talbot, S. J. (2001) Kaposi's sarcoma-associated herpesvirus vCyclin open reading frame contains an internal ribosome entry site. *J Virol* 75:1864-1869.
6. Bravo, D. A., Gleason, J. B., Sanchez, R. I., Roth, R. A., and Fuller, R. S. (1994) Accurate and efficient cleavage of the human insulin proreceptor by the human proprotein-processing protease furin. Characterization and kinetic parameters using the purified, secreted soluble protease expressed by a recombinant baculovirus. *J Biol Chem* 269:25830-25837
7. Chang, P. S., Lo, C. F., Wang, Y. C., and Kou, G. H. (1996) Identification of white spot syndrome associated baculovirus (WSSV) target organs in shrimp, *Penaeus monodon*, by in situ hybridization. *Dis Aquat Org* 27:131-139.
8. Chang, J., Yang, S. H., Cho, Y. G., Hwang, S. B., Hahn, Y. S., and Song, Y. C. (1998) Hepatitis C virus core from two different genotypes has an oncogenic potential but is not sufficient for transforming primary rat embryo fibroblasts in cooperation with the H-ras oncogene. *J Virol* 72:3060-3065.
9. Chen, L. L., Leu, J. H., Huang, C. J., Chou, C. M., Chen, S. M., Wang, C. H., Lo, C. F., and Kou, G. H. (2002a) Identification of a nucleocapsid protein (VP35) gene of shrimp white spot syndrome virus and characterization of the motif important for targeting VP35 to the nuclei of transfected insect cells. *Virology* 293:44-53.
10. Chen, L. L., Wang, H. C., Huang, C. J., Peng, S. E., Chen, Y. G., Lin, S. J., Chen, W. Y., Dai, C. F., Yu, H. T., Wang, C. H., Lo, C. F., and Kou, G. H. (2002b) Transcriptional analysis of the DNA polymerase gene of shrimp white spot syndrome virus. *Virology* 301:136-147.
11. Chou, H. Y., Huang, C. Y., Wang, C. H., Chiang, H. C., and Lo, C. F. (1995) Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. *Dis Aquat Org* 23:165-173.
12. Couch, J. A. (1974) An enzootic nuclear polyhedrosis virus of pink shrimp: ultrastructure, prevalence, and enhancement. *J Invertebr Pathol* 24:311-31.
13. Flegel, T. M., Sriurairatana, S., Wongteerasupaya, C., Boonsaeng, V., Panyim, S., and Withyachumrarnkul, W. (1995) Progress in characterization and control of yellow head virus of *Penaeus monodon*. In: Browdy CL, Hopkins JS, eds. *Swimming Through Troubled Water, Proceedings of the Special Session on Shrimp Farming*. World Aquaculture Society, Baton Rouge, LA, USA, 76-83.
14. Flegel TW. (1997) Special topic review: Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. *World J Microbiol Biotech* 13: 422-433.
15. Flint, M., Thomas, J. M., Maidens, C. M., Shotton, C., Levy, S., Barclay, W. S., and Mckeating, J. A. (1999) Functional analysis of cell surface expressed hepatitis C virus E2 glycoprotein. *J Virol* 73:6782-6790.
16. Gale, M. Jr., Tan, S. L., and Katze, M. G. (2000) Translational control of viral gene expression in eukaryotes. *Microbiol Mol Biol Rev* 64:239-280.
17. Geballe, A. P., and Mocarski, E. S., (1988) Translational control of cytomegalovirus gene expression is mediated by upstream AUG codons. *J Virol* 62:3334-3340
18. Gesteland, R. F., and Atkins, J. F. (1996) Recoding: dynamic reprogramming of translation. *Annu Rev Biochem* 65:741-768.
19. Hasson, K. W., Lightner, D. V., Poulos, B. T., Redman, R. M., White, B. L., and Brock, J. A. (1995) Taura syndrome in *Penaeus vannamei*: demonstration of a viral etiology. *Dis Aquat Org* 23:115-126.
20. Huang, J., Song, X. L., Yu, J., and Yang, C. H. (1995a) Baculoviral hypodermal and hematopoietic necrosis - study on the pathogen and pathology of the explosive epidemic disease of shrimp. *Mar Fish Res* 16:1-10.
21. Huang, C. C., Song, Y. L. (1999) Maternal transmission of immunity to white spot syndrome associated virus (WSSV) in shrimp (*Penaeus monodon*). *Dev Comp Immunol* 23:545-52.
22. Huang, J., Yu, J., Song, X. L., Kong, J., and Yang, C. H. (1995b) Studies on fine structure, nucleic acid, polypeptide and serology of hypodermal and hematopoietic necrosis baculovirus of penaeid shrimp. *Mar Fish Res* 16:11-23.
23. Inouye, K., Miwa, S., Oseko, N., Nakano, H., and Kimura, T. (1994) Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: Electron microscopic evidence of the causative virus. *Fish Pathol* 29:149-158.
24. Jean, F., Stella, K., Thomas, L., Liu, G., Xiang, Y., Reason, A. J., and Thomas, G. (1998) Alpha1-Antitrypsin Portland, a bioengineered serpin highly selective for furin: application as an antipathogenic agent. *Proc Natl Acad Sci USA*. 95:7293-7298.
25. Karunasagar, I., Otta, S. K., and Karunasagar, I. (1997) Histopathological and bacteriological study of white spot syndrome of *Penaeus monodon* along the west coast of India. *Aquaculture* 153:9-13.
26. Kurosaki, M., Enomoto, N., Marumo, F., and Sato, C. (1993) Rapid sequence variation of the hypervariable region of hepatitis C virus during the course of chronic infection. *Hepatology* 18:1293-1299.
27. Leu, J. H., Tsai, J. M., Wang, H. C., Wang, A. H., Wang, C. H., Kou, G. H., and Lo, C. F. (2005) The unique stacked rings in the nucleocapsid of the white spot syndrome virus virion are formed by the major structural protein VP664, the largest viral structural protein ever found. *J Virol* 79:140-149.
28. Lightner, D. V., Poulos, B. T., Redman, R. M., Mari, J., and Bonami, J. R. (1992) New developments in penaeid virology: Application of biotechnology in research and disease diagnosis for shrimp viruses of concern in the Americas. In: Fulks, W., and Main, K. (eds) *Proceedings of the Asian Interchange Program Workshop on the Diseases of Cultured Penaeid Shrimp*, Asian Interchange Program, The Oceanic Institute, Oahu, HI, 233-253.
29. Lightner DV. (1996) *A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp*. Society, Baton Rouge, LA: The World Aquaculture. 304
30. Lindenbach, B. D., and Rice, C. M. (2001) *Flaviviridae: the viruses and their replication*, pp. 991 – 1041 in D.M. Knipe, P.M. Howley, D.E. Griffin: *Fields Virology*, 4th ed. Lippincott – Williams & Wilkins, Philadelphia (USA).
31. Liu, W. J., Yu, H. T., Peng, S. E., Chang, Y. S., Pien, H. W., Lin, C. J., Huang, C. J., Tsai, M. F., Huang, C. J., Wang, C. H., Lin, J. Y., Lo, C. F., and Kou, G. H. (2001) Cloning, characterization, and phylogenetic analysis of a shrimp white spot syndrome virus gene

that encodes a protein kinase. *Virology* 289:362-377. 32. Liu, W., Wang, Y. T., Tian, D. S., Yin, Z. C., and Kwang, J. (2002) Detection of white spot syndrome virus (WSSV) of shrimp by means of monoclonal antibodies (MAbs) specific to an envelope protein (28 kDa). *Dis Aquat Organ*. 49:11-8. 33. Li, Z., Lin, Q., Chen, J., Wu, J. L., Lim, T. K., Loh, S. S., Tang, X., and Hew, C. L. (2007) Shotgun identification of structural proteome of shrimp white spot syndrome virus and iTRAQ differentiation of envelope and nucleocapsid subproteomes. *Mol Cell Proteomics*. *:*-*

34. Lo, C. F., Ho, C. H., Peng, S. E., Chen, C. H., Hsu, H. C., Chiu, Y. L., Chen, Y. T., Chang, C. F., Liu, K. F., Su, M. S., Wang, C. H., and Kou, G. H. (1996a) Infection of white spot syndrome associated virus (WSBV) in cultured and wild-caught shrimps, crabs and other arthropods. *Dis Aquat Org* 27:215-225. 35. Lo, C. F., Leu, J. H., Ho, C. H., Chen, C. H., Peng, S. E., Chen, Y. T., Chou, C. M., Yeh, P. Y., Huang, C. J., Chou, H. Y., Wang, C. H., and Kou, G. H. (1996b) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. *Dis Aquat Org* 25:133-141. 36. Lo, C. F., Ho, C. H., Chen, C. H., Liu, K. F., Chiu, Y. L., Yeh, P. Y., Peng, S. E., Hsu, H. C., Liu, H. C., Chang, C. F., Su, M. S., Wang, C. H., and Kou, G. H. (1997) Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. *Dis Aquat Org* 30:53-72. 37. Lo, C. F., and Kou, G. H. (1998) Virus associated white spot syndrome of shrimp in Taiwan: a review. *Fish Pathol* 33:365-371. 38. Lotz, J. M., Browdy, C. L., Carr, W. H., Frelief, P. F., and Lightner, D. V. (1995) USMSFP suggested procedures and guidelines for assuring the specific pathogen status of shrimp broodstock and seed. In: Browdy CL, Hopkins JS (eds) *Swimming Through Troubled Water, Proceedings the Special Session on Shrimp Farming, Aquaculture '95*. World Aquaculture Society, Baton Rouge, Louisiana, USA, 66-75. 39. Lo, S. Y., Masiarz, F., Hwang, S. B., Lai, H. H., and Ou, J. H. (1995) Differential subcellular localization of hepatitis C virus core gene products. *Virology* 213:455-461. 40. Marks, H., Mennens, M., Vlak, J. M., and van Hulten, M. C. (2003) Transcriptional analysis of the white spot syndrome virus major virion protein genes. *J Gen Virol* 84:1517-1523. 41. Mclauchlan, J. (2000) Properties of the hepatitis C virus core protein: a structural protein that modulates cellular processes. *J Viral Hepatitis* 7:2-14. 42. Momoyama, K., Hiraoka, M., Nakano, H., Koube, H., Inouye, A. K., and Oseko, N. (1994) Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: Histopathological study. *Fish Pathol* 29:141-148. 43. Moon, C. H., Do, J. W., Cha, S. J., Yoon, W. J., Kim, S. B., Ko, M. S., Park, M. A., Kim, J. W., Sohn, S. K., Lee, J. H., and Park, J. W. (2003) Highly conserved sequences of three major virion proteins of a Korean isolate of white spot syndrome virus (WSSV). *Dis Aquat Organ* 53:11-13. 44. Nadala, E. C. B., Tappy, L. M., and Loh, P. C. (1997) Yellow-head virus: a rhabdovirus-like pathogen of penaeid shrimp. *Dis Aquat Org* 31:141 – 146. 45. Nakano, H., Koube, H., Umezawa, S., Momoyama, K., Hiraoka, M., Inouye, K., and Oseko, N. (1994) Mass mortalities of cultured kuruma shrimp, in Japan in 1993: epizootiological survey and infection trials. *Fish Pathol* 29:135-139. 46. Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner A. J., Houghton, M., Rosa, D., Grandi, G., and Abrignani, S. (1998) Binding of hepatitis C virus to CD 81. *Science* 282:938-941. 47. Robalino, J., Payne, C., Parnell, P., Shepard, E., Grimes, A. C., Metz, A., Prior, S., Witteveldt, J., Vlak, J. M., Gross, P. S., Warr, G., and Browdy, C. L. (2006) Inactivation of White Spot Syndrome Virus (WSSV) by normal rabbit serum: implications for the role of the envelope protein VP28 in WSSV infection of shrimp. *Virus Res* 118:55-61. 48. Seok, S. H., Park, J. H., Cho, S. A., Baek, M. W., Lee, H. Y., Kim, D. J., and Park, J. H. (2004) Cloning and sequencing of envelope proteins (VP19, VP28) and nucleocapsid proteins (VP15, VP35) of a white spot syndrome virus isolate from Korean shrimp. *Dis Aquat Organ* 60:85-88. 49. Shimizu, Y. K., Igarashi, H., Kiyohara, T., Cabezon, T., Farci, P., Purcell, R. H., and Yoshikura, H. (1996) A hyperimmune serum against a synthetic peptide corresponding to the hypervariable region 1 of hepatitis C virus can prevent viral infection in cell cultures. *Virology* 223:409-412. 50. Tang, X., Wu, J., Sivaraman, J., and Hew, C. L. (2007) Crystal structures of major envelope proteins VP26 and VP28 from white spot syndrome virus shed light on their evolutionary relationship. *J Virol*. 81:6709-17. 51. Takahashi, Y., Itami, T., Kondo, M., Maeda, M., Fujii, R., Tomonaga, S., Supamattaya, K., and Boonyaratpalin, S. (1994) Electron microscopic evidence of bacilliform virus infection in kuruma shrimp (*Penaeus japonicus*). *Fish Pathol* 29:121-125. 52. Taylor, D. R., Shi, S. T., Romano, P. R., Barber, G. N., and Lai, M. M. C. (1999) Inhibition of the interferon inducible protein kinase PKR by HCV E2 protein. *Science* 285:107-110. 53. Tsai, J. M., Wang, H. C., Leu, J. H., Hsiao, H. H., Wang, A. H., Kou, G. H., and Lo, C. F. (2004) Genomic and proteomic analysis of thirty-nine structural proteins of shrimp white spot syndrome virus. *J Virol* 78:11360-11370. 54. Tsai, M. F., Lo, C. F., van Hulten, M. C., Tzeng, H. F., Chou, C. M., Huang, C. J., Wang, C. H., Lin, J. Y., Vlak, J. M., and Kou, G. H. (2000a) Transcriptional analysis of the ribonucleotide reductase genes of shrimp white spot syndrome virus. *Virology* 277:92-99. 55. Tsai, M. F., Yu, H. T., Tzeng, H. F., Leu, J. H., Chou, C. M., Huang, C. J., Wang, C. H., Lin, J. Y., Kou, G. H., and Lo, C. F. (2000b) Identification and characterization of a shrimp white spot syndrome virus (WSSV) gene that encodes a novel chimeric polypeptide of cellular-type thymidine kinase and thymidylate kinase. *Virology* 277:100-110. 56. Van Hulten, M. C., Goldbach, R. W., and Vlak, J. M. (2000) Three functionally diverged major structural proteins of white spot syndrome virus evolved by gene duplication. *J Gen Virol*. 81:2525-2529. 57. Van Etten, J. L., Graves, M. V., Muller, D. G., Boland, W., and Delaroque, N. (2002) Phycodnaviridae--large DNA algal viruses. *Arch Virol* 147:1479-1516. 58. Van Hulten, M. C., Reijns, M., Vermeesch, AM., Zandbergen, F., Vlak, J. M. (2002) Identification of VP19 and VP15 of white spot syndrome virus (WSSV) and glycosylation status of the WSSV major structural proteins. *J Gen Virol* 83:257-265. 59. Van Hulten, M. C., and Vlak, J. M. (2001) Identification and phylogeny of a protein kinase gene of white spot syndrome virus. *Virus Genes* 22:201-207. 60. Van Hulten, M. C., Witteveldt, J., Snippe, M., and Vlak, J. M. (2001) White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. *Virology*. Jul 285:228-233. 61. Wang, C. H., Lo, C. F., Leu, J. H., Chou, C. M., Yeh, P. Y., Chou, H. Y., E. T. M., Chang, C. F., Su, M. S., and Kou, G. H. (1995) Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. *Dis Aquat Org* 23:239-242. 62. Weiner, A. J., Geysen, H. M., Christopherson, C., Hall, J. E., Mason, T. J., Saracco, G., Bonino, F., Crawford, K., Marion, C. D., and Crawford, K. A. (1992) Evidence for immune selection of hepatitis C

virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infections. *Proc Nat Acad Sci USA* 89:3468-3472. 63. Witteveldt, J., Vermeesch, A. M., Langenhof, M., de Lang, A., Vlak, J. M., and van Hulten, M. C. (2005) Nucleocapsid protein VP15 is the basic DNA binding protein of white spot syndrome virus of shrimp. *Arch Virol*. 150:1121-33. 64. Witteveldt, J., Vlak, J. M., and van Hulten, M. C. (2006) Increased tolerance of *Litopenaeus vannamei* to white spot syndrome virus (WSSV) infection after oral application of the viral envelope protein VP28. *Dis Aquat Organ* 70:167-70. 65. Wongteerasupaya, C., Vickers, J. E., Sriurairatana, S., Nash, G. L., Akarajamorn, A., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyanchumnarnkul, B., and Flegel, T. W. (1995) A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in black tiger prawn *Penaeus monodon*. *Dis Aquat Org* 21:69-77. 66. Xu, Z., Choj, J., Yen, T. S. B., Lu, W., Strohecker, A., Govindarajan, S., Chien, D., Selby, M. J. and Ou, J.H. (2001) Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. *EMBO J* 20:3840-3848. 67. Yang, F., He, J., Lin, X., Li, Q., Pan, D., Zhang, X., and Xu, X. (2001) Complete genome sequence of the shrimp white spot bacilliform virus. *J Virol* 75:11811-11820. 68. Yi, G., Wang, Z., Qi, Y., Yao, L., Qian, J., and Hu, L. (2004) Vp28 of shrimp white spot syndrome virus is involved in the attachment and penetration into shrimp cells. *J Biochem Mol Biol* 37:726-734. 69. Yoganandhan, K., Syed, M. S., Narayanan, R. B., and Sahul, H. A. S. (2004) Production of polyclonal antiserum against recombinant VP28 protein and its application for the detection of white spot syndrome virus in crustaceans. *J Fish Dis* 27:517-522.