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Dna Profile of Sitophilus oryzae and S. zeamais In Rice and Corn Kernels

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Abstract: The problem in postharvest pests has to be taken seriously since the recent researches were not comprehensively resolved the problem. The researches on preferences, morphological characteristics, and DNA profile are required in order to strengthen the knowledge and the decision making in postharvest pest management, especially *Sitophilus* spp. The aims of this reseach was to analysis the DNA profile and develop the phylogeny tree of *S. oryzae* dan *S. zeamais* in rice and corn kernels. This research was emphasized on taxonomy and biological evolution through molecular biology approach. The method was used observational, descriptive, and experimental method. DNA analysis was conducted using the *Sofware MEGA (Molekular Evolutionary Gene Analysis) Versi 4.0.* The DNA sequencing data were confirmed by *Basic Local Alignment Search Tool (BLAST)* program before analysis. Sequencing parameters that analyzed were polymorphic site, genetic distance, and phylogenetic tree. Genetic distance was measured by *Kimura 2 parameter* method.
Results showed that the nucleotide bases of *S. zeamais* located at Gorontalo and *S. oryzae* at Manado tend to be different from those in other locations, although they were relatives (*intraspecific relationship*). The difference in nucleotide base of DNA sheet of *S. zeamais* located at Gorontalo with other Sitophilus was at 413 bp sheet that was adenine (A) turned into timine (T), *S. oryzae* located at Manado was at 158 bp sheet in which guanine (G) turned into adenine (A), and *Sitophilus* sp (brown in color) was at 200 bp sheet that is timine (T) turned into cytosine (C). Genetic distance of Gorontalo *S. zeamais* and Manado *S. oryzae* with other *S. oryzae* and *S. zeamais* was 0,002 and *Sitophilus* sp (brown in color) was 0.004. Genetic differences among individuals within populations of *S. oryzae* and *S. zeamais* from Manado, Kotamobagu, Talaud, and Gorontalo was the kinship within individual in species (*intraspecific relationship*). *Sitophilus* sp (brown in color) leads to interspecific relationship i.e. the genetic speciation or the new species was formed. Phenotype of *S. oryzae* and *S. zeamais* showed that those insects are different species, however, genotype of *S. oryzae* and *S. zeamais* demonstrated that they are relatives or the same species. Brown *Sitophilus* sp. on the phylogeny tree structure has led to the new speciation or new species formation and its name *Sitophilus* spn.
Keywords : Sitophilus oryzae, S. zeamais, Sitophilus sp (brown color), polymorphic site, genetic distance, phylogenitic tree construction.

Introduction

Sitophilus weevil is an important cosmopolitan pest which attacks postharvest commodity grains over the world[1] ; Rees in Cao[2]. This pest is included in the genus Sitophilus, family Curculionidae, and the order Coleoptera. It is known that there are two important species which invade postharvest commodity grains, especially rice and corn i.e. *S. oryzae* and *S. zeamais*[3,4] . According to Gillespie *et al.*[5] , the systematic and taxonomic researches are needed for the success of biological control of both pest types.

Weevil Sitophilus is a host of different kinds of bacteria endosymbiotic [6,2] . Endosymbiotic is an interspecific association event and takes a crucial role in many ecology events and evolutionary processes of living beings in nature[7]. The existence of Symbion and some metabolites in the beetle's body will remain stable as long as the existence of Symbion does not lead to a deficiency of food to beetles, and this endosymbiotic event is taking place in the metabolism process[7,6].

Metabolic processes will take place in accordance to the biological information which is stored in the form of genetic codes that are in the genetic material, i.e. DNA molecule (*deoxyribonucleic acid*). Genetic properties of living things are stored in the DNA strands which are nucleotides polymers. DNA is one type of amino acid that plays a role in passing the genetic information or genetic inheritance to the offspring of each individual. Nucleic acids are polymeric compounds that store all the genetic information. DNA is a polymer that is composed of three main components, i.e. a phosphate group, deoxyribose sugar, and a nitrogenous base. A monomer units of DNA consists of three components called nucleotides, thus DNA is classified as polynucleotide. DNA chain has width of 22-24 Å , while the length of a nucleotide unit is 3.3 Å and DNA can have millions nucleotides that are strung like a chain. For instance, the largest human chromosome consists of 220 million nucleotides [8,9].

DNA consists of two strands that are twisted to form a double helix structure. In the double helix structure, the orientation of the nucleotide chain on one strand is opposite to the other nucleotides strand orientation. This is known as antiparallel. Each strand consists of a main frame, as the main structure, and a nitrogenous base, which interacts with the one DNA strand in the helix. Both strands on the DNA double helix are united by hydrogen bonds between the bases which are contained on the second strand. The four bases that are found in DNA are adenine (denoted by A, cytosine (C), guanine (G), and thymine (T). Hydrogen bonded adenine with thymine, while guanine hydrogen bond with cytosine [8,10].

The discovery of Polymerase Chain Reaction (PCR) technique causes quite revolutionary changes in the various fields. The result of the application of this PCR technique is called DNA fingerprint which is a DNA fragments pattern picture of each individual, because each individual in the population has different DNA fingerprint. The DNAs which are commonly used in tests are mitochondrial DNA and cell nucleus DNA. The most accurate DNA for testing is the cell nucleus DNA because the cell nucleus cannot change while the DNA in mitochondrial can change because it is derived from the maternal lineage, which can change over offspring marriage [11,9].

Yuwono , clarify the shape and structure of the DNA or chromosome into a chemical macromolecular which is a character-inheriting substance (identity) of the parent to the child or the next generation. One piece (a fragment) of DNA double strand is composed of nucleotides polymer and each nucleotide consists of three components in the form of one molecule of nitrogen base, a five-carbon sugar molecule (deoxiribose), and a phosphate group. There are four molecules of DNA macromolecule building constituent nitrogen base, i.e. adenine, timine, guanine, and cytosine. Adenine (A) nitrogen molecule bases always pairs with Timine (T), while Guanine (G) pairs with cytosine (C). Arrangement or sequence of nucleotide bases affects the genetic makeup of each organism either individuals in population or between species[10].

One of methods to identify DNA polymorphism is Denaturing Gradient Gel Electrophoresis (DGGE) method. This technique can be used to separate different DNA molecules because of changes in one base nucleotide. The separation method is based on the observation that the DNA molecules that are different by a single base nucleotide have slightly different denaturation properties so that the molecules will migrate with different patterns in polyacrylamide gels which containing a linear gradient, DNA denaturator compounds. DGGE method can be used to detect approximately 50% of all single nucleotide changes that may occur in a DNA fragment to 1000 base pairs. In addition, this technique can also be used to detect the polymorphism that cannot be detected by other methods, such as RFLP (Restriction Fragment Length Polymorphisms) analysis[10].

Sheffield *et al.* (1990) in Yuwono[10] . have developed a method for amplifying genomic DNA to be used in DGGE analysis without having to use a DNA tracer that is by using denaturing gradient gel. There are two denaturing gradient gels, i.e.: (1) parallel gel, i.e. a gel containing DNA denaturator gradient that increase linearly from top to bottom, and (2) vertical gel, i.e. a gel containing DNA denaturator linear gradient from left to right to cut the gel. Parallel gels can be used to analyze more than one sample at a same gel, while the vertical gel can be used for polymorphism identification in a single individual in a pedigree. If polymorphism can be found in a DNA tracer with vertical gel method then polymorphism in a group of individuals can be tested using parallel DGGE method. The detailed research technique on genetic differences and finding out the origin of an organism is through gene duplication techniques through the use of reversed transcriptase enzyme called RT-PCR (Reverse Transcriptase - Polymerase Chain Reaction). Based on this, this research is so urgent to be conducted because each individual in a species have each DNA fingerprint. Besides, this research also wants to study the phylogeny tree construction of *S. oryzae* and *S. zeamais* that exist in North Sulawesi and Gorontalo.

The study aims to determine the DNA profile of *Sitophilus oryzae* and *S. zeamais* which include changes in nucleotide base pairs (polymorphic site), genetic distance of DNA bands, and Phylogenitic tree construction of S. oryzae and S. zeamais on 4 different geographical locations in North Sulawesi and Gorontalo.

Materials and Methode

The study is conducted in North Sulawesi and Gorontalo. Insect sampling is conducted in Gorontalo (GTO), Kotamobagu (KBG), Talaud (TLD) and Manado (MDO). Rearing for Sitophilus strain purification is conducted at the Laboratory of Entomology and Plant Pests, Faculty of Agriculture, UNSRAT, Manado in August to December 2010. DNA extraction and PCR amplification is carried out in the Laboratory of Biomedical and Animal Molecular Biology, Faculty of Veterinary, Udayana University, Bali and the sequencing analysis of DNA is carried in the Eijkman Institute, Jakarta on June to September 2011.

A. Materials and Tools

The materials and tools that are used in this research include:

a. Sitophilus Sample. *S. oryzae* and *S. zeamais* are collected from the field in the form of shelled rice and corn which has been identified attacked by pest. *S. oryzae* and *S. zeamais* are taken from Manado, Kotamobagu, Talaud and Gorontalo and reared in Laboratory of Pest and Plant Disease, Faculty of Agriculture, Unsrat, Manado for species purification. The purification of Sitophilus species (*S. oryzae* and *S. zeamais*) is conducted until the third generation (F-3). The Sitophilus sampling site may be seen at Figure 1.

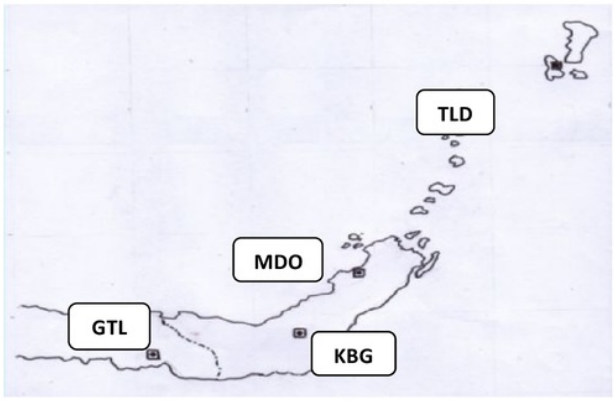


Figure 1. The *Sitophilus spp.* Sampling Site Map in North Sulawesi and Gorontalo Province. Description: MDO = Manado; TLD = Talaud; KBG = Kotamobagu; and GTL = Gorontalo.

b. Primary. The used primaries are three pairs of mutually overlapping primary. 1) One leads to the front, i.e. SOF 72 (5'-TGG ARC ATG ATC AGG AAT AGT RGG -3' and otherwise SOR (5'-AGA DAA TWG GGT CTC CKC CTC-3')[1] ; 2) One leads to the front, i.e. C1-J1709 (5'-AAT TGG WGG WTT YGG AAA YTG-3') and otherwise C1-J2353 (5'-GCT CGT GTA TCA ACG TCT ATW CC-3')[2] ; 3) One leads to the front, i.e. C1-J2183 (5'-CAA CAT TTA TTT TGA TTT TTT GG-3') and otherwise TL2-N3014 (5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3')[2] .

c. PCR (*Polymerase Chain Reaction).* The used tools for thermocycling process in DNA identification of *S. oryzae* and *S. zeamais*. Thermocyling is reaction sequence that occurs on extraction result DNA in PCR tool for 2 - 2.5 hours.

d. Genomic DNA Mini Kit (Invitrogen, PureLink™). Genomic DNA Mini Kit is a set of materials and equipment needed in the DNA extracts. Genomic DNA Mini Kit consists of: Genomic digestion buffer, proteinase K, RNase A, genomic lyses / binding buffer, elution buffer genomic, genomic wash buffer 1,

genomic wash buffer 2, ethanol, epemdorf tube, spin collum, cartridge, etc.

B. Research Method

a. DNA Extraction

DNA Sitophilus is extracted using genomic DNA Mini Kit (Invitrogen, PureLink™) following the DNA Extraction guidelines of Biomedicine and Molecular Biology Laboratory Animal Veterinary Faculty, Udayana University, Bali. *S. oryzae* and *S. zeamais* imago that are derived from each sampling location are taken of each and crushed in Eppendorf tubes using micropastle, added 180 µl of Genomic Digestion Buffer and homogenized again by using micropastle. The ependorf tube is closed, wrapped with aluminum foil and paraffin, and then boiled in hot water at a temperature of 100^{0}C for approximately 5 minutes and vortexed every 30 seconds. Chill in refrigerator at 8^{0}C for 10 minutes, add Proteinase K 20 µl, and then incubate in Oven at 55^{0}C for 2.5 hours and occasionally vortexed. Centrifuge at max speed for 3 minutes, then take the supernatant and fill into the new ependorf tube, add 20 µl of RNAse A, vortex and incubate for 2 minutes. Add 200 µl of Binding Buffer then vortex and add 200 µl of Ethanol, move to spin collum, centrifuge at 10,000 rpm for 1 minute. Discard flow (bottom layer fluid), move to the new spin collum, add 500 µl of Wash Buffer 1, and then centrifuge for 1 minute at 10,000 rpm. Discard flow, add 500 µl of Wash Buffer 2, centrifuge at max speed for minutes. Discard flow, move to 1.5 ml tube, add 100 µl of Elution Buffer, incubate for 1 minute and centrifuge at max speed for 1 minute. The extract result DNA is moved to the new spin collum and stored in freezer at –20^{0}C while waiting PCR amplification job.

b. *Reverse transcriptase polymerase chain reaction (RT-PCR)* Amplification

Three primary pairs of *Sitophilus* which are overlapping are used for the mitochondria DNA amplification. The first primary pair that leads to the front, i.e. SOF 72 (5'-TGG ARC ATG ATC AGG AAT AGT RGG-3' and otherwise SOR (5'-AGA DAA TWG GGT CTC CKC CTC-3') is used for CO1 amplification. The second primary pair that leads to the front, i.e. C1-J1709 (5'-AAT TGG WGG WTT YGG AAA YTG-3') and otherwise C1-N2353 (5'-GCT CGT GTA TCA ACG TCT ATW CC-3') is used for COI - C1 amplification. The third primary pair that leads to the front, i.e. C1-J2183 (5'-CAA CAT TTA TTT TGA TTT TTT GG-3') and otherwise TL2-N3014 (5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3') is used for COI - C1 - TL2 amplification. The PCR product is purified using Rapid PCR purification system (Marligen Biosciences, MD) and thinned with 30 µl of hot deionized water. RT-PCR is DNA amplification technique using enzyme and template which is complementer with target DNA. This test component consists of 5 µl of R-Mix (buffer, MgSo4, and dNTP), 0.6 µl of forward primary, 0.6 µl of backward primary, 2.55 µl of aquabidest, 0.25 µl of enzyme and 1 µl of DNA template sample. After all components are filled into the eppendorf tube, then it is put in *Thermocycler*. In *Thermocycler* (DNA cycler engine), the temperature has been programmed at 94°C for 7 minutes, that is pre-denaturation process which is DNA change process from the multiple fibers into a single fiber. Furthermore, 94°C for 45 seconds is Denaturation process that is multiple fibers that the release is rudimentary broken again so that it becomes single fiber. *Annealing* process is conducted at 52°C for 45 seconds, i.e. primary sticking process using DNA sample fiber. At the time of primary use, the sequence of both primaries' sequence should be not complementary to each other because it can result in primary dimmer. If the length of its primary is long enough, the temperature should be higher. Its elongation at 72 °C for one minute is primary lengthening process, so that it lengthens the DNA chain. The Taq Polymerase enzyme plays a role here. All of the above process is repeated for 35 cycles. The temperature of 72°C for 5 minutes is an improvement process of enzyme activity. After all process is completed, it continues to the electrophoresis process.

c. Electrophoresis

Electrophoresis is performed to determine the base length of the tested gene products through the marker. The electrophoresis process begins with the manufacture of the gel 1% that is 1 gram of agarose powder is added with 100 ml of TAE (Tris acetate EDTA), boiled while stirred occasionally. After the powder melts and slightly thickened, add 20 µl of EtBr (Ethidium Bromide), stir, and mold in the comb mold. After the gel hardens and then put on the electrophoresis engine. Ten to twenty percent of the PCR product is added by 1 µl of loading dye (Bromphenol-blue and Cyline Cyanol), inserted into each well on the gel. After that, it is runned by programming the electrophoresis machine at 100 volts for 30 minutes. Then, it is taken and the DNA visualization is conductor using transluminator ultraviolet (UV) and the results are documented using Polaroid cameras and films.

d. DNA Sequencing Analysis

Sequencing is conducted to find out the gene sequence that we want. The used primary at the amplification process is also used on this process. The detail of sequencing preparation process is as the following. A total of 20 µl of PCR product is added with 100 µl of PB buffer (*Binding buffer)* and moved to *catridge collum*, then centrifugated for 1 minute at 13,000 rpm. In *catridge*, PE buffer (*Wash of Buffer*) is added with 750 µl and centrifugated for 30-60 seconds at 6,000 rpm. *Catridge* is put into *spin collum* again and centrifugated for 1 minute at 13,000 rpm. After this, *Catridge* is put into ependorf tube again and added with 50 µl of EB buffer (*Elution Buffer)*. *Eluat* DNA is stored after being centrifugated for 1 minute. The pured PCR product is mixed with *Ready Reaction Mix* and *sequencing buffer 5x (Big Dye Terminator KIT)*, and 1 µl of planned primary. The cycle is performed at *Thermocycler* at 96°C for 3 minutes, 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 64°C for 4 minutes. A total of 5 µl of 125 mM EDTA and 60 µl ethanol 100 % solution is added into the *cycle sequencing* tube result. The compound is incubated at the room temperature for 15 minutes and then centrified at temperature of 4°C and velocity of 6,500 rpm for 30 minutes. Its sediment is added with 60 µl 70% ethanol and centrifuged at 13,000 rpm for 20 minutes, its supernatant is discarded and its pellet is treated with *dry air* for 10-15 minutes. The pellet is added with 20 µl of *Hi Dye* and vortexed for 1 minute. The mixture is heated using *Hot Block* at 95°C for 3 minutes, and then cooled with ice for 3 minutes. A total of 15 µl of the mixture is moved into the *plate sequencing* and put into *Try* in *Automatic DNA Sequencer*. Then, the DNA analysis is conducted.

e. Data Analysis

Nucleotide sequence of Sitophilus gene is aligned using Crustal W in the MEGA 4.0 program[12]. The analyzed data are the sequencing result confirmation with the Basic Local Alignment Search Tool (BLAST) program. The Sitophilus sequence result is compared with the other Sitophilus gene which is downloaded from GeneBank. The sequence parameters that are analyzed includes: Polimorfic Site, genetic distance and the origin of the tree (phylogenetic tree). The genetic distances are calculated using Kimura 2 parameter method . Based on the phylogeny tree, it can be known the genetic distance between species, and the evolution pattern of living things[13].

Results nd Discussion

The pool results of three primary kinds which are used on all DNA sample of extraction result give a quite good result (Figure 2).

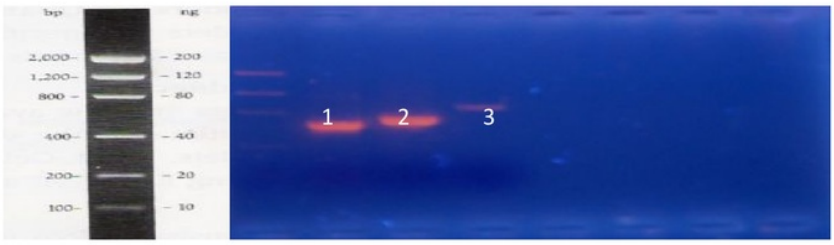


Figure 2. Pool Result DNA Tape on All DNA Samples of Extraction Result.

Where: A = DNA Mass ladder; B = DNA Tape of RT-PCR Result; 1 = First primary pair that leads to the front, i.e. SOF 72 ,(5'-TGG ARC ATG ATC AGG AAT AGT RGG-3' and otherwise SOR (5'-AGA DAA TWG GGT CTC CKC CTC-3'); 2. = The second pair that leads to the front, i.e. C1-J1709 (5'-AAT TGG WGG WTT YGG AAA YTG-3') and otherwise C1-N2353 (5'-GCT CGT GTA TCA ACG TCT ATW CC-3'); 3. = The third primary pair that leads to the front, i.e. C1-J2183 (5'-CAA CAT TTA TTT TGA TTT TTT GG-3') and otherwise TL2-N3014 (5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3').

The pool result (Figure 2) shows that two primary pairs, i.e. first primary pair (SOF 72 (5'-TGG ARC ATG ATC AGG AAT AGT RGG-3' and the otherwise SOR, (5'-AGA DAA TWG GGT CTC CKC CTC-3) and the second primary pair (C1-J1709 (5'-AAT TGG WGG WTT YGG AAA YTG-3') and the otherwise C1-N2353 (5'-GCT CGT GTA TCA ACG TCT ATW CC-3')) can catch Sitophilus DNA well and give clear DNA tape image. The third primary pair (C1-J2183 (5'-CAA CAT TTA TTT TGA TTT TTT GG-3') and otherwise TL2-N3014 (5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3') gives poor DNA tape result or obtain less

clear DNA tape image. Thus, only two primary pairs, i.e. the first primary pair and the second primary pairs are suitable to be used in PCR amplification of the DNA of *S. oryzae* and *S. zeamais* and *Sitophilus sp* (brown).

The RT-PCR amplification result on the *DNA* Sample of *S. oryzae* and *S. zeamais* that are collected from Manado, Talaud, Kotamobagu and Gorontalo, and brown *Sitophilus* sp. which is from *S. zeamais* population in Talaud can be seen at Figure 3.

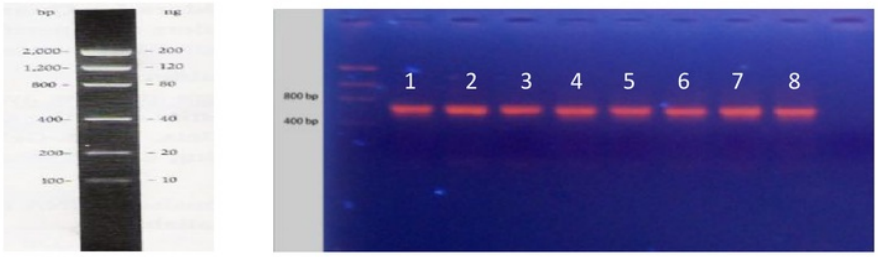


Figure 3. DNA Tapes of *Sitophilus oryzae*, *Sitophilus zeamais*, and *Sitophlus* sp. RT-PCR Result.

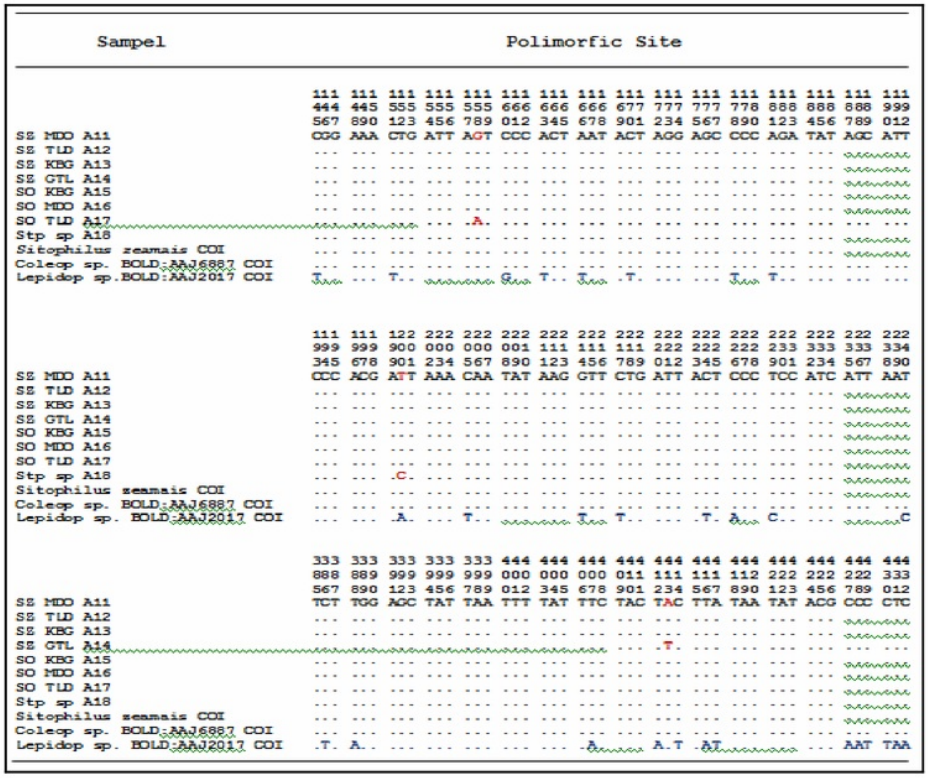
Where: A = DNA mass lader; B = DNA Tape of RT-PCR Result; 1 = SZ MDO A11 (*S. zeamais* from Manado); 2 = SZ TLD A12 (*S. zeamais* from Talaud); 3 = SZ KBG A13 (*S. zeamais* from Kotamobagu); 4 = SZ GTL A14 (*S. zeamais* from Gorontalo); 5 = SO KTG A15 (*S. oryzae* from Kotamobagu); 6 = SO MDO A16 (*S.oryzae* from Manado); 7 = SO TLD A17 (*Sitophilus oryzae* from Talaud); 8 = *Sitophilus* sp. A18 (brown *Sitophilus* sp.).

The PCR amplification result on eight imago sample of *Sitophilus* spp. Shows that there is only one primary pair which obtain clear DNA tape, i.e. the first primary pair (SOF 72 (5'-TGG ARC ATG ATC AGG AAT AGT RGG-3' and otherwise SOR (5'-AGA DAA TWG GGT CTC CKC CTC-3)) (designed by Manueke[1]. The PCR result shows that based on DNA Mass Lader, the area of DNA tapes of *S. oryzae, S. zeamais,* and (brown) *Sitophilus sp.* are about at 400 – 800 bp.

The sequence analysis result shows that DNA tapes of *S. oryzae* and *S. zeamais* are at area of or the length of DNA tapes are 552 bp and have nucleotide bases sequence that tends to be similar (homologous). Homologous DNA means DNA tapes that have similarities in shape, size/length, and their nucleotide bases. The samples that have homologous DNA sequences are a sample number 1 (SZ A11 MDO), i.e. *S. zeamais* from Manado, number 2 (SZ TLD A12) i.e. *S. zeamais* from Talaud, number 3 (SZ BECs A13) i.e. *S. zeamais* from Kotamobagu location, number 5 (SO BECs A15) i.e. *S. oryzae* from Kotamobagu, and sample number 16 (SO MDO A16) i.e. *S. oryzae* from Manado. Samples that have difference in nucleotide bases are sample number 14 (SZ GTL A14) i.e. *S. zeamais* from Gorontalo, number 17 (SO TLD A17) i.e. *S. oryzae* from Talaud and sample number 18 (Stp sp A18) i.e. brown *Sitophilus sp.* which derived from a population of S. zeamais from Talaud.

The nucleotide base and DNA tape area difference of *S. zeamais* form Gorontalo, *S. oryzae* from Talaud and brown *Sitophilus* sp are presented on Table 1.

Table 1. The Different Area and Nucleotide at DNA Tape of *Sitophilus oryzae, Sitophilus zeamais,* and *Sitophilus sp.* Sequence Analysis Result.



Sampel	Polimorfic Site
	111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111
	444 445 555 555 555 666 666 666 677 777 777 778 888 888 888 999
	567 890 123 456 789 012 345 678 901 234 567 890 123 456 789 012
SZ MDO A11	CGG AAA CTG ATT AGT CCC ACT AAT ACT AGG AGC CCC AGA TAT AGC ATT
SZ TLD A12	... ...
SZ KBG A13	... ...
SZ GTL A14	... ...
SO KBG A15	... ...
SO MDO A16	... ...
SO TLD A17	... ... .A. ... ...
Stp sp A18	... ...
Sitophilus zeamais COI	... ...
Coleop sp. BOLD:AAJ6887 COI	... ...
Lepidop sp.BOLD:AAJ2017 COI	T.. ... T.. ... ... G.. T.. T.. .T. ... ... T.. T.. ... ...
	111 111 122 222 222 222 222 222 222 222 222 222 222 222 222 222
	999 999 900 000 000 001 111 111 111 222 222 222 233 333 333 334
	345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890
SZ MDO A11	CCC ACG ATT AAA CAA TAT AAG GTT CTG ATT ACT CCC TCC ATC ATT AAT
SZ TLD A12	... ...
SZ KBG A13	... ...
SZ GTL A14	... ...
SO KBG A15	... ...
SO MDO A16	... ...
SO TLD A17	... ...
Stp sp A18	... ... .C. ... ...
Sitophilus zeamais COI	... ...
Coleop sp. BOLD:AAJ6887 COI	... ...
Lepidop sp. BOLD:AAJ2017 COI	... ... .A. ... T.. ... ... T.. T.. ... .T. A.. C.. C
	333 333 333 333 333 444 444 444 444 444 444 444 444 444 444 444
	888 889 999 999 999 000 000 000 011 111 111 112 222 222 222 333
	567 890 123 456 789 012 345 678 901 234 567 890 123 456 789 012
SZ MDO A11	TCT TGG AGC TAT TAA TTT TAT TTC TAC TAC TTA TAA TAT ACG CCC CTC
SZ TLD A12	... ...
SZ KBG A13	... ...
SZ GTL A14	... ...
SO KBG A15	... ...
SO MDO A16	... ...
SO TLD A17	... ...
Stp sp A18	... ...
Sitophilus zeamais COI	... ...
Coleop sp. BOLD:AAJ6887 COI	... ...
Lepidop sp. BOLD:AAJ2017 COI	.T. A.. ... ... .A. ... A.T .AT ... ... AAT TAA

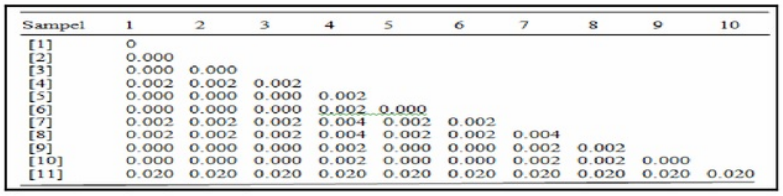
Where: 1. SZ MDO A11 = *S. zeamais* from Manado; 2. SZ TLD A12 = *Sitophilus zeamais* from Talaud; 3. SZ KBG A13 = *Sitophilus zeamais* from Kotamobagu; 4.SZ GTL A14 = *Sitophilus zeamais* from Gorontalo; 5. SO KTG A15 = *Sitophilus oryzae* from Kotamobagu; 6. SO MDO A16 = *Sitophilus oryzae* from Manado; 7. SO TLD A17 = *Sitophilus oryzae* from Talaud; 8. Stp sp A18 = brown *Sitophilus* sp.).

Gene bank involves 3 comparator genes, i.e. *S. zeamais* COI, Coleoptera sp. Bold: AAJ6887 COI, and Lepidoptera sp. Bold: AAJ2017 COI. *S. zeamais* COI and Coleoptera sp. Bold: AAJ6887 COI are *intraspesific relationship* comparators and Lepidoptera sp. Bold AAJ2017 COI is *interspesific relationship* comparator.

The nucleotide base difference in *S. oryzae* and *S. zeamais* population is at *S. zeamais* from Gorontalo at number 413 base pairs, i.e. Adenine (A) base becomes Timine (T), *S. oryzae* from Talaud at number 158 base pairs, i.e. Guanine (G) base becomes Adenine (A), and *Sitophilus* sp. which is derived from *S. zeamais* population from Talaud at number 200 base pair sequence, i.e. Timine (T) base becomes Cytosine (C) base.

The *genetic distance* between individuals in *S. oryzae* and *S. zeamais* population in North Sulawesi and Gorontalo are presented on Table 2.

Table 2. *Genetic Distance* of Nucleotide Base on DNA Tape of *Sitophilus zeanais* and *Sitophilus oryzae* in North Sulawesi and Gorontalo.



Sampel	1	2	3	4	5	6	7	8	9	10
[1]	0									
[2]	0.000									
[3]	0.000	0.000								
[4]	0.002	0.002	0.002							
[5]	0.000	0.000	0.000	0.002						
[6]	0.000	0.000	0.000	0.002	0.000					
[7]	0.002	0.002	0.002	0.004	0.002	0.002				
[8]	0.002	0.002	0.002	0.004	0.002	0.002	0.004			
[9]	0.000	0.000	0.000	0.002	0.000	0.000	0.002	0.002		
[10]	0.000	0.000	0.000	0.002	0.000	0.000	0.002	0.002	0.000	
[11]	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020

Where: [1]=SZ MDO A11; [2]=SZ TLD A12; [3]=SZ KBG A13; [4]=SZ GTL A14; [5]=SO KBG A15; [6]=SO MDO A16; [7]=SO TLD A17; [8]=Stp sp A18; [9]=*Sitophilus zeamais* COI; [10]=Coleoptera sp BOLD:AAJ6887 COI; [11]=Lepidoptera sp BOLD:AAJ2017 COI.

The data on Table 2 shows that between *S. zeamais* from Manado and *S. zeamais* from Talaud, *S. oryzae* from Manado and *S. zeamais* from Kotamobagu, *S. zeamais* from Talaud and *S. zeamais* from Kotamobagu, *S. zeamais* from Manado and *S. oryzae* from Kotamobagu, *S. zeamais* from Talaud and *S. oryzae* from Kotamobagu, *S. zeamais* from Kotamobagu and *S. oryzae* from Kotamobagu, *S. zemais* from Manado and *S. oryzae* from Manado, *S. zeamais* from Talaud and *S. oryzae* from Manado, *S. zeamais* from Kotamobagu and *S. oryzae* from Manado, and *S. oryzae* from Kotamobagu and *S. oryzae* from Manado quantitatively do not have genetical distance.

S. zeamais from Manado and *S. zeamais* from Gorontalo, *S. zeamais* from Talaud and *S. zeamais* from Gorontalo, *S. zeamais* from Kotamobagu and *S. zeamais* from Gorontalo, *S. oryzae* from Kotamobagu and *S. zeamais* from Gorontalo, *S. oryzae* from Manado and *S. zeamais* from Gorontalo, *S. oryzae* from Manado, *S. oryzae* from Talaud and *S. zeamais* from Manado, *S. oryzae* from Talaud and *S. zeamais* from Talaud, *S. oryzae* from Talaud and *S. zeamais* from Kotamobagu, *S. oryzae* from Talaud and *S. oryzae* from Kotamobagu, *S. oryzae* from Talaud and *S. oryzae* from Manado, *S. zeamais* from Manado and *Sitophilus* sp., *S. zeamais* from Talaud and *Sitophilus* sp., *S. zeamais* from Kotamobagu and *Sitophilus* sp., *S. oryzae* from Kotamobagu and *Sitophilus* sp., and *S. oryzae* from Manado and *Sitophilus* sp., have genetical distance of 0.002. *S. zeamais* from Gorontalo and *S. oryzae* from Talaud, *S. zeamais* from Gorontalo and *Sitophilus* sp., and *S. oryzae* from Talaud and *Sitophilus* sp. have genetical distance of 0.004.

Nucleotide base change on organism in nature occurs in genetic transformation process through *"P-Element Transformation System"* that occurs in male and female parent mating (hybridization) and mutation. Gilbert states that genetic expression on organisms is the result of genetic transformation that occurs during the process of genetic transcription[14]. Transcription is the process of copying the genetic code that is in the DNA sequence into RNA molecules and is a process that started the expression of genetic traits that would later appear as a phenotype. According to Atkinson, *et al.*, genetic inheritance occurs in a system of biological organisms called genetic transformation system that enables the genetic changes in an organism[15]. Changes in the genetic trait may occur because of the mating between individuals within populations and gene mutations. Genetic changes depend on the time length of individual group separation with the origin population and physical and food environmental factors.

The phylogeny tree construction of *S. oryzae*, *S. zeamais*, and brown *Sitophilus* sp from Manado, Kotamobagu, Talaud, and Gorontalo is presented on Figure 4.

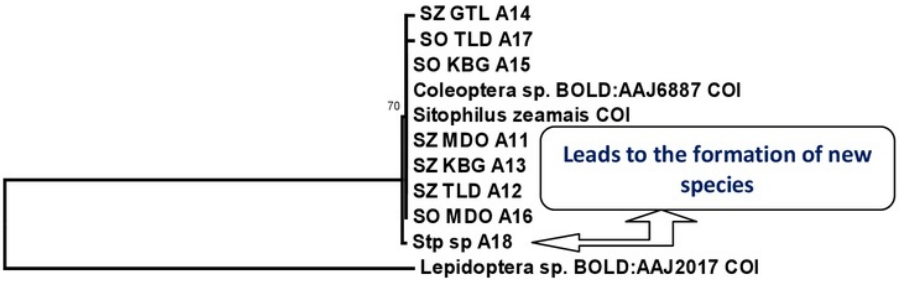


Figure 4. The Phylogeny Tree construction of *Sitophilus oryzae* and *Sitophilus zeamais* Using Neighbor joining Kimura 2 Parameter Method[13].

Where: SZ MDO A11 = *S. zeamais* from Manado; SZ TLD A12 = *S. zeamais* from Talaud; SZ KBG A13 = *S. zeamais* from Kotamobagu; SZ GTL A14 = *S. zeamais* from Gorontalo; SO KTG A15 = *S. oryzae* from Kotamobagu; SO MDO A16 = *S. oryzae* from Manado; SO TLD A17 = *S.oryzae* from Talaud; Stp sp A18 = *Sitophilus* sp. (brown); Coleoptera sp. Bold: AAJ6887 COI, *Sitophilus zeamais* COI and Lepidoptera sp. Bold: AAJ2017 COI = comparator gene from Gene Bank.

The pylogeny tree construction of *S. zeamais* and *S. oryzae* shows that all *Sitophilus* samples include in a clade, except *Sitophilus* sp which is derived from *S. zeamais* population from lokasi Talaud. This shows that *S. zeamais* from Manado, *S. zeamais* from Talaud, *S. zeamais* from Kotamobagu, *S. zeamais* from Gorontalo, *S.*

oryzae from Kotamobagu and *S. oryzae* from Manado genetically still a relative (a species), which includes in the intraspecific relationship. While (brown) Sitophilus sp has formed a new clade (interspecific relationships), that has formed a new speciation or a new species.

The occurrence of speciation or genetic changes in *Sitophilus* sp from Talaud may be due to several things as follows: 1) This insect has been isolated away from the original population; 2) The existence of the separation barrier between its origin population, i.e. the ocean; 3) Environment or habitat that is different with the native populations. Boughey[16] and Shoonhoven *et al.* [17] states that there are three theories or hypothesis of the speciation occurrence in insects, i.e.: 1). Allopatrik speciation theory (the geographic speciation theory), that is a group of individuals which is separated from its origin population because of some barrier, such as rivers, lakes, or sea in a long time. Under these conditions, the reproductive evolution mechanisms occur to the organisms which are separated from their native populations; 2). The parapatric speciation theory, that is the speciation in separated group of individuals from the population due to move to new habitats even though there is no physical barrier. Speciation like this happens because of the host or the new feed. Occupancy in the new habitat will result a barrier to gene flow between populations in new habitats. Parapatric speciation is common in organisms that are not winged or slow in movement; 3). Sympatrik speciation theory, that is speciation which occurs when reproductive isolation occurs in the range of a population before any differentiation of the two species is detected or two groups of individuals from the a population occupy two different host. Such speciation usually occurs in parasites or parasitoids that have specific host.

According Marjorie [18] and Laffin *et al.* [19], the insect that has a narrow spread scale has low genetic diversity. De Sale *et al.*[20] suggest that a low level of diversity can also be caused by a short residence time in a new area / habitat that is not enough time to accumulate genetic distance. Species that spread in a vast area show a fairly high genetic variation[21].

Conclusion

Based on the research result and discussion, it may be concluded that:

1. The DNA tapes of *S. oryzae* and *S. zeamaie* are at DNA tape area or length of 552 bp and have nucleotide base sequences that tends to be similar (homologous).
2. The nucleotide base of *S. zeamais* from Gorontalo and *S. oryzae* from Talaud tend to be different with the other population of *S. oryzae* and *S. zeamais*, but still an *intraspecific relationship*.
3. The nucleotide base difference on DNA tape of *S. zeamais* from Gorontalo and the other Sitophilus is at the tape area of 413 bp, that is adenine (A) nucleotide base changes into timine (T), *S. oryzae* from Talaud's is at the tape area of 158 bp, that is guanine (G) nucleotide changes into adenine (A), and (brown) *Sitophilus* sp's is at the tape area of 200 bp, that is timine (T) nucleotide base changes into cytosine (C).
4. The genetic distance between *S. zeamais* and *S. oryzae* in North Sulawesi and Gorontalo is 0.002, and (brown) *Sitophilus* sp with *S. zeamais* and *S. oryzae* is 0.004.
5. Based on the phylogeny tree construction, (brown) *Sitophilus* sp. has genetically formed new speciation or already led to the new species formation (*interspecific relationship*), or is new species so that its name is *Sitophilus* spn.

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