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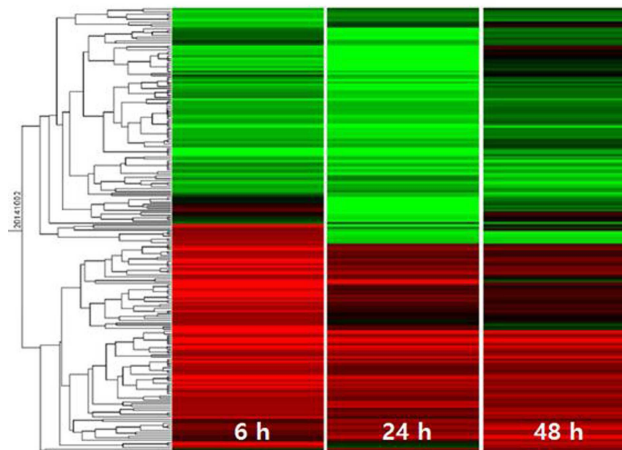
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(54) 발명의 명칭 디클로페낙 노출에 대응하는 히드라 유전자 및 이를 이용한 수생태계 환경오염 진단 방법

(57) 요약

본 발명은 디클로페낙(Diclofenac) 노출에 대응하는 히드라(*Hydra magnipapillata*) 유전자 및 이를 이용하여 수생태계 환경오염을 진단하는 방법에 관한 것으로, 구체적으로 DDBJ/EMBL/NCBI 유전자 데이터베이스에 축적되어 있는 히드라의 유전자 정보로부터 17,000여 개의 개체(singleton)를 추출한 후, 이에 대한 oligo-probe를 디자인하여, 이를 탑재한 17K Hydra Express Gene Microarray(HEGEM)을 완성하였고, 6, 24, 및 48시간 동안 디클로페낙(Diclofenac) 노출에 의해 발현량이 2배 이상 증가하거나 감소한 유전자 51종을 발굴함으로써, 상기 유전자를 디클로페낙 노출 여부를 확인할 수 있는 바이오마커로 사용하고, 이는 수생태계에서 디클로페낙의 노출 여부를 확인하는데 유용하게 사용될 수 있다.

대표도 - 도1



[7.0 µg/L, 1/1,000 72 h LC<sub>50</sub>]

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## 명세서

### 청구범위

#### 청구항 1

서열번호 1 내지 51로 기재되는 모든 유전자 각각의 핵산 서열의 올리고뉴클레오티드 전부 또는 그의 상보가닥 분자가 집적된, 시료내 디클로페낙(Diclofenac) 노출 여부 검출용 마이크로어레이 칩(microarray chip).

#### 청구항 2

제 1항에 있어서, 상기 유전자는 히드라(*Hydra magnipapillata*)로부터 유래된 것을 특징으로 하는 디클로페낙 노출 여부 검출용 마이크로어레이 칩.

#### 청구항 3

제 1항에 있어서, 서열번호 1 내지 40으로 기재되는 유전자로 구성된 군으로부터 선택되는 유전자는 디클로페낙 노출에 대응하여 발현이 증가하는 것을 특징으로 하는 디클로페낙 노출 여부 검출용 마이크로어레이 칩.

#### 청구항 4

제 1항에 있어서, 서열번호 41 내지 51로 기재되는 유전자로 구성된 군으로부터 선택되는 유전자는 디클로페낙 노출에 대응하여 발현이 감소하는 것을 특징으로 하는 디클로페낙 노출 여부 검출용 마이크로어레이 칩.

#### 청구항 5

- 1) 피검 시료에 노출된 실험군의 히드라와, 정상 대조군의 히드라에서 각각 RNA를 분리하는 단계;
- 2) 단계 1)의 실험군 및 대조군의 RNA로부터 cDNA를 합성하면서 실험군과 대조군을 각기 다른 형광물질로 표지하는 단계;
- 3) 단계 2)의 각기 다른 형광물질로 표지된 cDNA를 제 1항의 마이크로어레이 칩과 혼성화시키는 단계;
- 4) 반응한 마이크로어레이 칩을 분석하는 단계; 및
- 5) 분석한 데이터에서 제 1항의 마이크로어레이 칩에 집적된 유전자 발현 정도를 대조군과 비교하여 확인하는 단계를 포함하는, 시료내 디클로페낙(Diclofenac) 노출 여부 검출 방법.

#### 청구항 6

제 5항에 있어서, 상기 시료는 생체시료, 식품시료, 화학시료, 공업시료, 임상시료 및 환경시료로 구성된 군으로부터 선택되는 어느 하나인 것을 특징으로 하는 디클로페낙 노출 여부 검출 방법.

#### 청구항 7

제 5항에 있어서, 상기 단계 2)의 형광물질은 Cy3, Cy5, FITC(poly L-lysine-fluorescein isothiocyanate), RITC(rhodamine-B-isothiocyanate) 및 로다민(rhodamine)으로 이루어진 군으로부터 선택되는 어느 하나인 것을 특징으로 하는 디클로페낙 노출 여부 검출 방법.

**청구항 8**

- 1) 피검시료에 노출된 실험군의 히드라(*Hydra magnipapillata*)와, 정상 대조군의 히드라에서 각각 RNA를 분리하는 단계;
- 2) 단계 1)의 RNA를, 서열번호 1 내지 51로 기재되는 각각의 유전자에 상보적이고 유전자를 증폭할 수 있는 프라이머 쌍을 모두 사용하여 정량 실시간 RT-PCR(Quantitative real-time reverse transcript polymerase chain reaction, qRT-PCR)을 수행하는 단계; 및
- 3) 단계 2)의 유전자 산물을 대조군과 비교하여 발현 정도를 확인하는 단계를 포함하는, 시료내 디클로페낙(Diclofenac) 노출 여부 검출 방법.

**청구항 9**

제 8항에 있어서, 상기 시료는 생체시료, 식품시료, 화학시료, 공업시료, 임상시료 및 환경시료로 구성된 군으로부터 선택되는 어느 하나인 것을 특징으로 하는 디클로페낙 노출 여부 검출 방법.

**청구항 10**

제 1항의 마이크로어레이 칩을 포함하는 디클로페낙(Diclofenac) 노출 여부 검출용 키트.

**청구항 11**

제 10항에 있어서, 스트렙타비딘-알칼리 탈인화효소 접합물질(streptavidin-like phosphatase conjugate), 화학형광물질(chemifluorescence) 및 화학발광물질(chemiluminescent)로 이루어진 형광물질군으로부터 선택되는 어느 하나를 추가적으로 포함하는 것을 특징으로 하는 디클로페낙 노출 여부 검출용 키트.

**청구항 12**

제 10항에 있어서, 혼성화에 사용되는 완충용액, RNA로부터 cDNA(complementary DNA)를 합성하기 위한 역전사효소, dNTPs(deoxynucleotide triphosphates) 및 rNTPs(ribonucleotide triphosphates, 사전 혼합형 또는 분리 공급형), 표식시약, 및 세척 완충용액으로 이루어진 반응 시약군으로부터 선택되는 어느 하나를 추가적으로 포함하는 것을 특징으로 하는 디클로페낙 노출 여부 검출용 키트.

**청구항 13**

서열번호 1 내지 51로 기재되는 각각의 유전자에 상보적이고 유전자를 증폭할 수 있는 프라이머 쌍을 모두 포함하는 디클로페낙(Diclofenac) 노출 여부 검출용 키트.

**발명의 설명**

**기술 분야**

[0001] 본 발명은 디클로페낙(Diclofenac) 노출에 대응하는 히드라(*Hydra magnipapillata*) 유전자 및 이를 이용하여 수생태계 환경오염을 진단하는 방법에 관한 것이다.

**배경 기술**

[0003] 디클로페낙(Diclofenac)은 비스테로이드성 항염증제(nonsteroidal anti-inflammatory drug, NSAID)로서, 관절염, 요통, 통풍 등의 치료를 위한 통증, 염증, 발열을 경감시키기 위해 사용한다. 그러나 디클로페낙은 심혈관

계 위험을 증가시키는 것으로 알려져 있고, 특히 노인들에게는 문제를 일으킬 수 있다고 한다. 전국 각 도시의 하수종말처리장 수질을 분석한 결과, 무분별하게 버려지고 있는 불용의약품으로 인한 콜레스테롤 저하제, 소염 및 해열 진통제 등이 검출되었다. 이런 의약품들은 인간뿐 아니라 동물과 농산물 생산, 수산물 양식에도 광범위하게 사용되고 있으며, 사람이나 동물에게 투여된 후 그 일부가 그대로 또는 생체 내에서 대사체로 변환되어 소변이나 대변으로 배설되어 환경 속으로 들어가게 된다. 또한 가정에서 사용하지 않거나 사용하다가 남은 약들은 쓰레기통, 변기에 버려지고 제약회사에서 생산되어 유통기한을 넘긴 의약품은 폐기되어야 하는데 그대로 환경에 버려지는 경우도 있어서 수질 등 환경오염으로 인하여 사람의 건강에 영향을 미치거나, 생태계를 교란할 경우가 있다. 이러한 이유로, 최근 환경에 잔류하는 의약품들이 새로운 오염물질로 거론되고 있으며, 사용과 폐기에 따른 지속적 방출로 인하여 하천 및 토양오염을 가중시킨다는 여러 연구 결과가 발표되고 있다.

[0005] 히드라(*Hydra magnipapillata*)는 신경세포를 갖고 있는 최초의 다세포 동물로서, 생물 진화의 연구에도 매우 중요한 위치를 차지하고 있다. 이배엽성 동물로서 번식은 무성 및 유성생식에 의하며, 뛰어난 재생능력을 갖고 있다. 히드라에 대한 선행 연구로는 형태형성과 관련된 신호전달계(Hobmayer 등, 2000, Nature 407: 186-189; Arvizu 등, 2006, Differentiation 74: 305-312; Augustin 등, 2006, Dev. Biol. 296: 62-70; Kaesbauer 등, 2007, Dev. Biol. 303: 376-390), 재생과 관련된 신호 및 신호전달계(Bode, 2003, Dev. Dyn. 226: 225-236; Fujisawa, 2003, Dev. Dyn. 226: 182-189; Holstein 등, 2003, Dev. Dyn. 226: 257-267), 세포분화의 신호전달과 관련된 연구(Thomsen 등, 2004, Mech. Dev. 121: 195-204; Philipp 등, 2005, Gene Expr. Patterns, 5: 397-402) 및 조직의 fate 결정 신호(Bielen 등, 2007, Development, 134: 4187-4197) 등이 알려져 있다. 이러한 신호전달계는 진화과정을 통해 모든 동물에 공통적으로 존재하므로, 히드라에서의 신호전달계 이상을 다른 동물에도 외삽할 수 있다. 대부분의 동물에서 초기발생과정에만 발현하는 다양한 유전자들이 히드라에서는 성체에서도 그발현이 유지된다. 따라서, 히드라를 이용하면 시기적인 제한을 받지 않고 이와 관련된 신호전달계의 연구가 가능하다. 또한, 히드라에서 각 세포 형에 대한 분자 마커들이 알려져 있어, 세포분화에 대한 연구도 가능하다. 유전자 및 단백질 발현의 위치화(localization)을 위한 in situ hybridization(ISH) 및 immunohistochemistry(IHC)법도 정립되어 있다. 현재 히드라의 160,000 클론의 ESTs 정보 및 genome data(Chapman 등, 2010, Nature 464: 592-596)가 DB화되어 있어, 다양한 신호전달계에 속하는 유전자들에 대한 정보 검색이 가능하다.

[0007] 이에, 본 발명자들은 디클로페낙 노출에 대한 특이 유전자후보의 확보 및 검출을 위해, DDBJ/EMBL/NCBI 유전자 데이터베이스에 축적되어 있는 히드라의 유전자 정보로부터 17,000여 개의 개체(singleton)를 추출한 후, 이에 대한 oligo-probe를 디자인하였으며, 이를 탑재한 17K Hydra Express Gene Microarray(HEGEM)을 완성하여, 디클로페낙 노출에 의해 발현량이 2배 이상 증가하거나 감소한 히드라 유래의 유전자 51종을 발굴함으로써, 이를 통해 상기 유전자를 디클로페낙 노출 여부를 확인할 수 있는 바이오마커로 사용하고, 이를 이용하여 디클로페낙의 노출 여부를 확인할 수 있음을 밝힘으로써 본 발명을 완성하였다.

**발명의 내용**

**해결하려는 과제**

[0009] 본 발명의 목적은 디클로페낙(Diclofenac) 노출에 대응하여 발현량이 변화된 히드라(*Hydra magnipapillata*) 유전자 및 이를 이용한 디클로페낙 노출 여부를 확인하는 방법을 제공하는 것이다.

**과제의 해결 수단**

[0011] 상기 목적을 달성하기 위하여, 본 발명은 서열번호 1 내지 51로 기재되는 모든 유전자 각각의 핵산 서열의 올리고뉴클레오티드 전부 또는 그의 상보가닥 분자가 집적된, 시료내 디클로페낙(Diclofenac) 노출 여부 검출용 마이크로어레이 칩(microarray chip)을 제공한다.

[0012] 또한, 본 발명은

- [0013] 1) 피검 시료에 노출된 실험군의 히드라와, 정상 대조군의 히드라에서 각각 RNA를 분리하는 단계;
- [0014] 2) 단계 1)의 실험군 및 대조군의 RNA로부터 cDNA를 합성하면서 실험군과 대조군을 각기 다른 형광물질로 표지하는 단계;
- [0015] 3) 단계 2)의 각기 다른 형광물질로 표지된 cDNA를 제 1항의 마이크로어레이 칩과 혼성화시키는 단계;

- [0016] 4) 반응한 마이크로어레이 칩을 분석하는 단계; 및
- [0017] 5) 분석한 데이터에서 제 1항의 마이크로어레이 칩에 집적된 유전자 발현 정도를 대조군과 비교하여 확인하는 단계를 포함하는, 시료내 디클로페낙(Diclofenac) 노출 여부 검출 방법을 제공한다.
- [0018] 또한, 본 발명은
- [0019] 1) 피검 시료에 노출된 실험군의 히드라(*Hydra magnipalillata*)와, 정상 대조군의 히드라에서 각각 RNA를 분리하는 단계;
- [0020] 2) 단계 1)의 RNA를, 서열번호 1 내지 51로 기재되는 각각의 유전자에 상보적이고 유전자를 증폭할 수 있는 프라이머 쌍을 모두 사용하여 정량 실시간 RT-PCR(Quantitative real-time reverse transcript polymerase chain reaction, qRT-PCR)을 수행하는 단계; 및
- [0021] 3) 단계 2)의 유전자 산물을 대조군과 비교하여 발현 정도를 확인하는 단계를 포함하는, 시료내 디클로페낙(Diclofenac) 노출 여부 검출 방법을 제공한다.
- [0022] 또한, 본 발명은 상기 마이크로어레이 칩을 포함하는 디클로페낙(Diclofenac) 노출 여부 검출용 키트를 제공한다.
- [0023] 아울러, 본 발명은 서열번호 1 내지 51로 기재되는 각각의 유전자에 상보적이고 유전자를 증폭할 수 있는 프라이머 쌍을 모두 포함하는 디클로페낙(Diclofenac) 노출 여부 검출용 키트를 제공한다.

**발명의 효과**

- [0025] 본 발명에서, DDBJ/EMBL/NCBI 유전자 데이터베이스에 축적되어 있는 유전자 정보로부터 히드라(*Hydra magnipapillata*)의 17,000여 개의 개체(singleton)를 추출한 후, 이에 대한 oligo-probe를 디자인하였으며, 이를 탑재한 17K Hydra Express Gene Microarray(HEGEM)을 완성하여, 디클로페낙(Diclofenac) 노출에 의해 발현량이 2배 이상 증가하거나 감소한 유전자 51종을 발굴하여, 상기 유전자를 디클로페낙 노출 여부를 확인할 수 있는 바이오마커로 사용하고, 이를 이용하여 디클로페낙의 노출 여부를 확인하는 방법으로써 유용하게 사용될 수 있다.

**도면의 간단한 설명**

- [0027] 도 1은 디클로페낙에 노출한 히드라의 차등 발현 유전자 프로파일링 Hierachial clustering 결과를 나타낸 도이다. 72시간에서 LC<sub>50</sub>의 약 1/100에 해당하는 7.0 µg/L(ppb)에 6시간, 24시간, 및 48시간 동안 노출하였다.

**발명을 실시하기 위한 구체적인 내용**

- [0029] 이하, 본 발명을 상세히 설명한다.
- [0031] 본 발명은 디클로페낙(Diclofenac) 노출에 대응하여 발현이 변화하는 히드라(*Hydra magnipalillata*) 유래의 유전자를 발굴하여, 상기 디클로페낙 노출에 대하여 발현량이 변화하는 히드라 유래 유전자를 집적한 마이크로어레이 칩을 디클로페낙 노출 여부 검출 및 수생태계 오염 상태를 진단하는데 이용할 수 있다.
- [0033] 본 발명은 서열번호 1 내지 51로 기재되는 모든 유전자 각각의 핵산 서열의 올리고뉴클레오티드 전부 또는 그의 상보가닥 분자가 집적된, 시료내 디클로페낙 노출 여부 검출용 마이크로어레이 칩(microarray chip)을 제공한다.
- [0034] 상기 유전자는 히드라(*Hydra magnipalillata*)로부터 유래된 것이 바람직하고, 서열번호 1 내지 40으로 기재되는 유전자로 구성된 군으로부터 선택되는 유전자는 디클로페낙 6, 24 및 48시간 노출에 의해 정상 대조군에 비해 발현량이 2배 이상 증가하는 것이 바람직하며, 서열번호 41 내지 51로 기재되는 유전자로 구성된 군으로부터 선택되는 유전자는 디클로페낙 6, 24 및 48시간 노출에 의해 정상 대조군에 비해 발현량이 2배 이상 감소되는 유전자인 것이 바람직하다.
- [0035] 상기 디클로페낙 노출 여부 검출용 마이크로어레이 칩은 당업자에게 알려진 방법으로 제작할 수 있다. 상기 마이크로어레이 칩을 제작하는 방법은 하기와 같다. 상기 탐색된 유전자를 프로브로 이용하여 마이크로어레이 칩의 기관상에 고정화시키기 위해 파이조일렉트릭(piezoelectric) 방식을 이용한 마이크로피펫팅(micropipetting)법 또는 핀(pin) 형태의 스폿터(spotter)를 이용한 방법 등을 사용하는 것이 바람직하나 이에 한정되지 않는



다. 상기 마이크로어레이 칩의 기판은 아미노-실란(amino-silane), 폴리-L-라이신(poly-L-lysine) 및 알데히드(aldehyde)로 이루어진 군에서 선택되는 하나의 활성기가 코팅된 것이 바람직하나 이에 한정되지 않는다. 또한, 상기 기판은 슬라이드 글라스, 플라스틱, 금속, 실리콘, 나일론 막, 및 니트로셀룰로스 막으로 이루어진 군에서 선택될 수 있으나 이에 한정되지 않는다.

- [0037] 또한, 본 발명은
- [0038] 1) 피검 시료에 노출된 실험군의 히드라와, 정상 대조군의 히드라에서 각각 RNA를 분리하는 단계;
- [0039] 2) 단계 1)의 실험군 및 대조군의 RNA로부터 cDNA를 합성하면서 실험군과 대조군을 각기 다른 형광물질로 표시하는 단계;
- [0040] 3) 단계 2)의 각기 다른 형광물질로 표시된 cDNA를 제 1항의 마이크로어레이 칩과 혼성화시키는 단계;
- [0041] 4) 반응한 마이크로어레이 칩을 분석하는 단계; 및
- [0042] 5) 분석한 데이터에서 제 1항의 마이크로어레이 칩에 집적된 유전자 발현 정도를 대조군과 비교하여 확인하는 단계를 포함하는, 시료내 디클로페낙(Diclofenac) 노출 여부 검출 방법을 제공한다.
- [0043] 상기 시료는 생체시료, 식품시료, 화학시료, 공업시료, 임상시료 및 환경시료로 구성된 군으로부터 선택되는 어느 하나인 것이 바람직하고, 상기 단계 2)의 형광물질은 Cy3, Cy5, FITC(poly L-lysine-fluorescein isothiocyanate), RITC(rhodamine-B-isothiocyanate) 및 로다민(rhodamine)으로 이루어진 군으로부터 선택되는 어느 하나인 것이 바람직하다.
- [0045] 또한, 본 발명은
- [0046] 1) 피검 시료에 노출된 실험군의 히드라(*Hydra magnipapillata*)와, 정상 대조군의 히드라에서 각각 RNA를 분리하는 단계;
- [0047] 2) 단계 1)의 RNA를, 서열번호 1 내지 51로 기재되는 각각의 유전자에 상보적이고 유전자를 증폭할 수 있는 프라이머 쌍을 모두 사용하여 정량 실시간 RT-PCR(Quantitative real-time reverse transcript polymerase chain reaction, qRT-PCR)을 수행하는 단계; 및
- [0048] 3) 단계 2)의 유전자 산물을 대조군과 비교하여 발현 정도를 확인하는 단계를 포함하는, 시료내 디클로페낙(Diclofenac) 노출 여부 검출 방법을 제공한다.
- [0049] 상기 시료는 생체시료, 식품시료, 화학시료, 공업시료, 임상시료 및 환경시료로 구성된 군으로부터 선택되는 어느 하나인 것이 바람직하다.
- [0051] 또한, 본 발명은 상기 마이크로어레이 칩을 포함하는 디클로페낙(Diclofenac) 노출 여부 검출용 키트(kit)를 제공한다.
- [0052] 상기 키트는 스트렙타비딘-알칼리 탈인화효소 접합물질(streptavidin-like phosphatase conjugate), 화학형광물질(chemifluorescence) 및 화학발광물질(chemiluminescent)로 이루어진 형광물질군으로부터 선택되는 어느 하나를 추가적으로 포함하는 것이 바람직하고, 혼성화에 사용되는 완충용액, RNA로부터 cDNA(complementary DNA)를 합성하기 위한 역전사효소, dNTPs(deoxynucleotide triphosphates) 및 rNTPs(ribonucleotide triphosphates, 사전 혼합형 또는 분리 공급형), 표식시약, 및 세척 완충용액으로 이루어진 반응 시약군으로부터 선택되는 어느 하나를 추가적으로 포함하는 것이 바람직하다.
- [0054] 아울러, 본 발명은 서열번호 1 내지 51로 기재되는 각각의 유전자에 상보적이고 유전자를 증폭할 수 있는 프라이머 쌍을 모두 포함하는 디클로페낙(Diclofenac) 노출 여부 검출용 키트를 제공한다.
- [0056] 본 발명의 구체적인 실시예에서, 본 발명자들은 디클로페낙 노출에 대응하는 히드라(*Hydra magnipapillata*) 유래의 유전자를 발굴하기 위하여, DDBJ/EMBL/NCBI 유전자 데이터베이스에 축적되어 있는 유전자 정보로부터 히드라의 17,000여 개의 개체(singleton)를 추출한 후, 이에 대한 oligo-probe를 디자인하여, 이를 탑재한 17K Hydra Express Gene Microarray(HEGEM)을 완성하였고, 6, 24, 및 48시간 동안 디클로페낙(Diclofenac) 노출에 의해 발현량이 2배 이상 증가하거나 감소한 유전자 51종을 선별하였다(표 2 및 표 3 참조).
- [0057] 따라서, 상기 유전자들을 포함하는 바이오마커를 프로브로 이용하여 피검체와 대조군의 RNA를 추출하여 상기 마커 유전자들의 발현 변화를 비교분석함으로써, 피검체가 디클로페낙에 노출되었는지 여부를 확인할 수 있다. 즉, 만약 피검체가 디클로페낙에 노출되었다면, 표 2에 기재되어 있는 유전자들의 발현량이 대조군에 비해 피검

체에서 증가한 것으로 나타날 것이고, 표 3에 기재되어 있는 유전자들의 발현량은 대조군에 비해 피검체에서 감소한 것으로 나타날 것이므로, 이를 이용하여 피검체의 디클로페낙의 노출 여부를 확인할 수 있다.

[0059] 이하, 본 발명을 실시예 및 실험예에 의해서 상세히 설명한다.

[0060] 단, 하기 실시예 및 실험예는 본 발명을 예시하기 위한 것일 뿐, 본 발명이 하기 실시예 및 실험예에 의해서 한정되는 것은 아니다.

[0062] <실시예 1> 특이 유전자 후보군의 분리 및 동정

[0063] <1-1> 히드라의 배양

[0064] 히드라(Hydra magnipapillata)의 야생형(wild strain) 105는 1 mM NaCl, 1 mM CaCl<sub>2</sub>, 0.1 mM KCl, 0.1 mM MgSO<sub>4</sub>, 1 mM Tris(hydroxymethyl) aminoethane (pH7.6)에 배양하였다. 수온은 20℃로 고정하였으며, 이틀에 한번 갓 부화한 Artemia 유생을 먹이로 공급하였다. 먹이 공급 후, 수 시간 이후에 배양액을 교환하였다.

[0066] <1-2> 디클로페낙 노출 조건

[0067] 히드라에 디클로페낙을 노출하는 조건을 확립하기 위해, 디클로페낙에 대한 반수치사농도(Lethal concentration 50, LC<sub>50</sub>)를 결정하였다(표 1).

표 1

노출 시간(시)	LC <sub>50</sub> (mg/L)
24	13.02
48	8.15
72	7.05

[0070] 상기 결과를 바탕으로, 차등발현 유전자 검출을 위한 노출 농도는 72시간의 LC<sub>50</sub> 값(7.05 mg/L)의 약 1/1,000에 해당하는 7.0 µg/L로 결정하고, 히드라 20개체에 각각 6, 24, 및 48시간 동안 노출하였다.

[0072] <1-3> RNA 추출 및 cDNA 합성

[0073] 각 노출군의 RNA 추출에는 Tri-reagent(Molecular Research Center Inc.)를 이용하였다. 추출된 RNA를 주형으로 역전사효소를 이용하여 cDNA를 합성하였다.

[0075] <1-4> Microarray 실험

[0076] 마이크로어레이(microarray) 실험을 위하여, 형광물질(Cy5 및 Cy3)이 라벨링된 cRNA 시료를 Qiagen PCR purification kit을 사용하여 정제하고, 증류수로 용출하였다. 그런 다음, 정제된 형광표지-cDNA 시료를 hybridization buffer(3×SSC, 0.3% SDS, 50% formamide, 20 µg Cot-1 DNA, 20 µg yeast tRNA)에 첨가한 후, microcon YM-30으로 농축하여 hybridization mixture를 만들었다. Hybridization mixture를 95℃로 3분 동안 가열하여 변성시키고 12,000×g에서 30초간 원심분리하며 온도를 낮추었다.

[0077] 그 후, 제조된 히드라 마이크로어레이에 커버슬립(cover slip)을 덮고, 변성시킨 hybridization mixture를 파이펫으로 주입한 후, GT-Hyb Chamber에 넣고 65℃에서 16시간 동안 반응시켜, Hybridization을 수행하였다. 다음으로, chamber에서 마이크로어레이를 꺼내어 세척한 후, 마이크로어레이를 spin하여 건조한 후 scanning할 때까지 암소에서 보관하였다.

[0078] 실험이 완료된 히드라 마이크로어레이를 Axon GenePix 4000B scanner(Axon Instrument, USA)를 사용하여 스캔하였다. GenePix Pro 6.0 프로그램을 이용하여, 스캔한 도면으로부터 각 spot을 gridding file을 이용하여 그리딩(griding)하였고, 이를 정량하여 각 spot의 Cy5/Cy3 세기(intensity) 및 비율 등의 분석 값이 포함된 GPR file을 얻었다.

[0079] 상기 GenePix Pro Program에서 얻어진 GPR file로부터, 분석 프로그램인 GeneSpring 7.3.1 (Agilent Technologies, USA)를 이용하여 분석을 수행하였다. 정규화(Normalization)는 LOWESS (locally weighted regression scatterplot smoothing)를 이용하여 수행하였고, Reliable gene은 중앙값(median)의 합이 기본(background) 값보다 낮거나, 각 픽셀(pixel) 값의 표준편차가 유의하지 않은 spot을 flag-out함으로써 유의한



유전자를 얻었다. Significant genes는 정규화한 중앙값이 2배 이상 차이를 보이는 spot을 선택하여 선별하였다.

[0081] <실험예 1> 특이유전자후보군의 선별

[0082] 디클로페낙 노출실험은 독립적으로 3회 실시하였고, 각 실험결과 중 통계적으로 유의하게 발현량이 변화된 유전자를 확인하여, 이들 중 발현량이 2배 이상 변화되는 유전자들을 선별하였다.

[0083] 그 결과, 도 1의 유전자 프로파일링에 나타난 바와 같이, 6시간 노출군에서 30종의 유전자(증가 25종, 감소 5종); 24시간 노출군에서 20종의 유전자(증가 11종, 감소 9종); 48시간 노출군에서 31종(증가 27종, 감소 4종)의 유전자가 발현이 변화되는 것으로 분석되었다(도 1).

[0084] 이들 중 중복되는 유전자들을 제외하면, 총 51종(증가 40종, 표 2; 감소 11종, 표 3)의 유전자들이 디클로페낙 노출에 의해 발현량이 변화되는 것으로 파악되었으며, 이들 유전자들은 디클로페낙 노출을 확인할 수 있는 바이오마커로 유용하게 사용할 수 있음을 확인하였다.

표 2

[0086] 디클로페낙 6, 24 및 48시간 노출군에서 발현량이 2배 이상 증가되는 주요 유전자 목록

서열번호	유전자
1	5-azacytidine induced 1, mRNA
2	Adal protein, mRNA
3	ADP-ribosylation factor-like 11, mRNA
4	Alpha-crystallin B chain, mRNA
5	arginyltransferase 1, mRNA
6	ATP-binding cassette, sub-family C (CFTR/MRP), member 2, mRNA
7	ATP-dependent DNA helicase PIF1, mRNA
8	chondroitin 4-sulfotransferase, mRNA
9	crinkled CG7595-PB, mRNA
10	Cysteine-rich protein 1, mRNA
11	erythrocyte membrane protein PFEMP3, mRNA
12	Ferric reductase like transmembrane component family protein, partial mRNA
13	GCN1 general control of amino-acid synthesis 1-like 1, partial mRNA
14	heat shock protein , partial mRNA
15	heat shock protein 90, alpha (cytosolic), class A member 1, mRNA
16	heat shock protein, mRNA
17	heparanase, mRNA
18	HMBA-inducible, mRNA
19	hydra Na channel 3 (hynac3), mRNA
20	Hydroxymethylglutaryl-CoA lyase, mitochondrial, mRNA
21	Late histone H2A.2.2, mRNA
22	lens intrinsic membrane protein 2.1, mRNA
23	Mib1 protein, partial mRNA
24	mitochondrial RNA ligase 2, mRNA
25	Mitogen-activated protein kinase-activated protein kinase 5, partial mRNA
26	mucin 1, cell surface associated, mRNA
27	Niemann-Pick disease, type C2 , mRNA
28	oxidative stress protein, mRNA
29	phosphatidylinositol glycan, class B, partial mRNA
30	Probable voltage-dependent N-type calcium channel subunit alpha-1B (Voltage-gated calcium channel subunit alpha Cav2.2) (DOE-4), partial mRNA
31	protein tyrosine phosphatase, non-receptor type 14, partial mRNA
32	radial spoke head 10 homolog B2, mRNA
33	Sedoheptulokinase , partial mRNA
34	small heat shock protein, partial mRNA
35	Sox10, mRNA
36	testis expressed gene 264, mRNA
37	Tumor necrosis factor, alpha-induced protein 8-like protein, mRNA
38	U2-associated SR140 protein, partial mRNA

39	WntX2, mRNA
40	WW domain-containing oxidoreductase, partial mRNA

**표 3**

[0088]

디클로페낙 6, 24 및 48 h 노출군에서 발현량이 2배 이상 감소되는 주요 유전자 목록

서열번호	유전자
41	casanova, mRNA
42	histone H2B, mRNA
43	peroxidase ppod11, partial mRNA
44	polycystic kidney disease 2, mRNA
45	Polycystic kidney disease 2-like 1 protein, partial mRNA
46	Sox17 alpha, mRNA
47	SRY-box containing gene 32, mRNA
48	stalk protein, mRNA
49	tetratricopeptide repeat domain 29, partial mRNA
50	thrombospondin type 1 repeat-containing protein 2, mRNA
51	tyrosine kinase receptor, mRNA

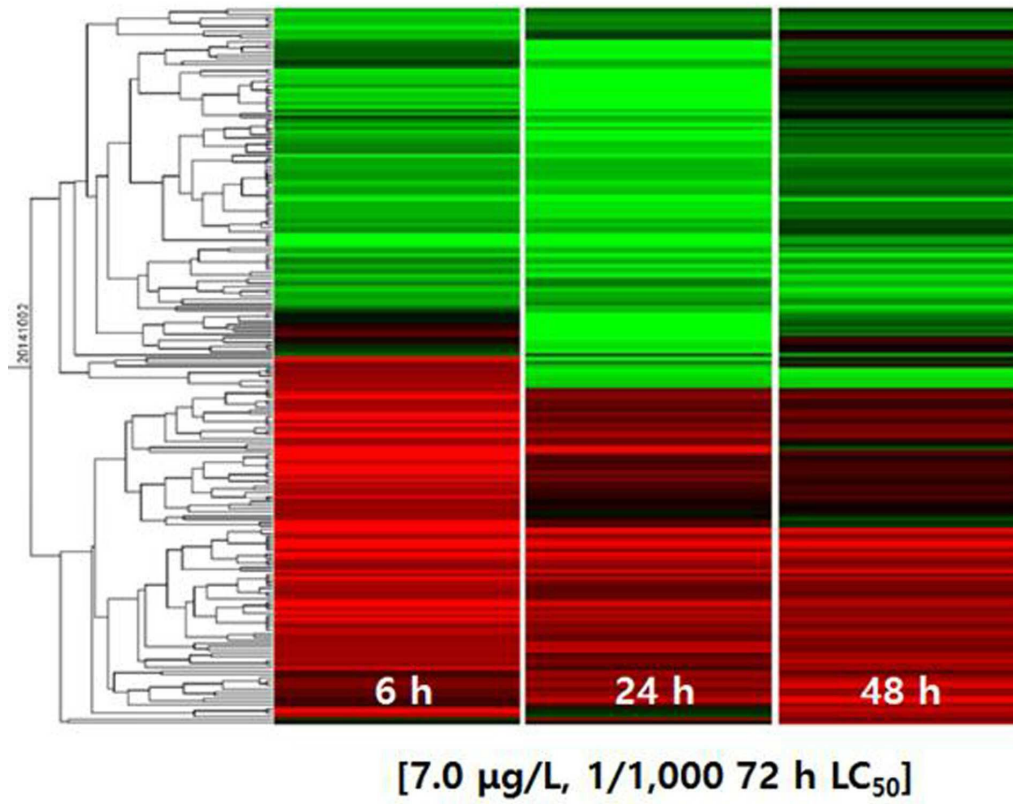
**산업상 이용가능성**

[0090]

본 발명에서 제시한 디클로페낙(Diclofenac) 노출에 대응하는 히드라(*Hydra magnipalillata*)의 유전자는 수생태계의 내분비계장애물질 오염 및 이에 따른 수생태계의 상황을 모니터링하고 진단하기 위한 바이오센서의 성분으로 유용하게 사용될 수 있다. 아울러, 제시된 유전자들의 기능에 의거하여 본 생물의 대사/생리변화를 구체화함으로써 앞으로 일어날 수도 있는 병리적 현상을 예측할 수 있는 생체지표 및 센서로 이용할 수 있으며, 수생태계의 스트레스원 검출 또는 건강 진단방법에 효과적으로 이용될 수 있다.

도면

도면1



서열목록

- <110> Korea Institute of Ocean Science and Technology
- <120> Diclofenac responsive genes in Hydra magnipapillata and the method for diagnosing aquatic environment pollution using the same
- <130> 2015P-12-049
- <160> 51
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aatcacataa atattgatgt tacaacacag tttatattgt tgctactatc attgtaaata 360

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<211> 784

<212> RNA

<213> Hydra magnipapillata

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gaatacaaaa taacttatca taatggagcc attattaatc atattaattt ttattatagg 180

gaacggata tgtttacaag tcacatcgat aaacttaaac aatcattcaa ttgaaataaa 240

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<210> 33

<211> 3160

<212> RNA

<213> Hydra magnipapillata

<400> 33

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<210> 34

<211> 813

<212> RNA

<213> Hydra magnipapillata

<400> 34

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<211> 1067

<212> RNA

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<210> 36

<211> 755

<212> RNA

<213> Hydra magnipapillata

<400> 36

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<210> 38

<211> 408  
 <212> RNA  
 <213> Hydra magnipapillata  
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 <211> 729  
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<213> Hydra magnipapillata  
 <400> 40

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 <400> 41

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 aactcatttt tgctttgggc aaaatctgta aggaaaaatt tttcaaatga taatcctaata 180  
 ctaaccaaca ctgaaataag tagagtgcta gggaaagtct ggaaagaaat gagtgaagtt 240  
 gagaaattgc cgtttattca aagtgcaaag tgccttcgaa ctaaattttt aatgattat 300  
 ccaaattatc aatacttgtg caaaaaacgg aaattcaatc aagtaataa tacaagtttt 360

tccaaaatgg cgtccaaact aggaagtaat gatttaaatg catacgaaat ttttaaatgt 420  
 ttaaacatga gtgaaattat agaacacaaa gatttgaaaa gctttggcta ctttcaaagt 480  
 gtattcactg cttatcaaaa cgaaagaaac gacaatcaaa atatattttt gcctgaagtt 540  
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 aaaaatataa gtgacagttt tgatgaaaat caacaaatgc aaaaagaaaa aactacgctg 660  
 cactactatt cagatgaaac acaacatttt aatcaaaatt cctttgacat aatagcgaa 720  
 gaagatttta atcaaaataa tcaataaaa gaaagagaag atgttattga acttgactct 780

gaattaagag aattttttca gtctcttgag aaaggttttg gttattatga atga 834

<210> 42

<211> 491

<212> RNA

<213> Hydra magnipapillata

<400> 42

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 tgcattcccga tgcggagtt tctgtctaaag cgatgagcat catgaattcg ttcgtgaacg 240  
 acatttttga aaggatcgcg tctgaagctt cgcgactcgc ctttcaaac aaaaagtcta 300

caatctcttc acgtgaaatt caaacagecg tgcgcctctt acttcccgtt gaattggcaa 360  
 aacacgcagt gagcgaaggc accaaagctg tcaccaagta cacgagcagc aagtagacgc 420  
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<210> 43

<211> 803

<212> RNA

<213> Hydra magnipapillata

<400> 43

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 cttttgaaat tcgtttcact aacgcacaaa catttgcat aaatcacat gcaaatggta 180  
 agtttgcag tgcaggtaat ggtggaata acttgctcat tgcaataaa gaccacgctg 240  
 cagtatggga gactttcacc ttagttccaa aataggagc ctttgattt aaatcaaatg 300  
 gtaatgcaa gttagtact gctgaagaag caggaaacaa accacttgca gctaactgca 360  
 ttgttcttga cgtctgggaa atgttcagct tagtctacgt ctggccatca gtacacaaag 420  
 tggcaataaa agcattggtt aatggtttat ttgtatgtgc tgaaaatgcc ggaaagcaat 480  
 cattaattgc taacagaggc caaatagggc cttgggaaac atttgaaatt cgcttcacta 540  
  
 acccccaaac atttactctc aaatctcttg caaacggcaa attggtttgc gcagaaaata 600  
 atggaaaatc tcctcttatt gctaataagag aacttatgg accatgggag acctttacgc 660  
 tggttcaaaa taaagaagga ttgctttca aatcacacgc aaatggaaaa ttagtgactg 720  
 ctgaaaacgc aggtcatagt aacctaattg ccaaccgca taatctgat atttgggaaa 780  
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<210> 44

<211> 2013

<212> RNA

<213> Hydra magnipapillata

<400> 44

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 aatgagaaca taatttacga aatgaagt gcaaattta actcgtaca aaaatatttt 180  
 tcgttaaac acaccaacc acctatgcca ctaatttccg ttgcttttaa tgaacctgc 240  
 aaaaataatg tagacaaagg tataatacca gatataaacg atgagacgtt aataaaatac 300  
 ttctctgac acaacaaaa ggataaagac cacaaaat t gatcgacca tcattattta 360  
 aacgaactac tcttatctaa cataagcacc aataatacag accgatatgg acagagtgtg 420  
 atgcatgagg tggctagagc ttgggatact gaagttgcaa aacttctatt aaaacatgtt 480  
  
 ctagctgcag agcttgatag gtcatttgca gcaaatatt taattgaaa tggagcagat 540  
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 cacagcatag atcgaaatc acgcacagag cactttaact taaattgtct tactttacca 660

ataaacgaga catcgtgttt tgcaaagtca ccttttagaga tcacagtgga aatgaaacaa 720  
 tacgaaatth tattacacc ggcaatgaat gaactaataa aagttaaag ggcaaagttt 780  
 ggaaaatgta gtgctctaaa aagtattgct gctaattttg cacttgtaat attttggaca 840  
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 tggggaccta ttgtcgagat tatagcagta gacacattag caataaatat tttattggaa 960  
 gtaaaagatt tctataaatc tctaaccagg tttaaaaagt ataaaaaatg gcgagaaaaa 1020  
 gaaattcgta aagatttaaa atattgtcat aaaaaatggc cagaagaacg tacttactta 1080  
 aaacaagaga tacgagagct caaaaactcc aagctatcat atataaaaga ttattggaac 1140  
 atttttgact gggttactta ttttttgatg gcattcagta tatctttaca ttaattgaa 1200  
 atggcgagac aaaacaagta ttcatgaca acaaaaaaat attcagcctc agctctaag 1260  
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 <211> 1397  
 <212> RNA  
 <213> Hydra magnipapillata  
 <400> 45  
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 actcacctat thtacctca actatgccat ggagatatca aacatggcaa gaacttgatg 180

ggtatccata tacagcaaat ttagacacct actatggtgg aggttatggt atagaaatct 240  
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 gacaaacacg agctgtaata atagagtttg ctttgtttaa tgctgctacc aattatntta 360  
 gcatggtcac aatggccttg gaatttcctg cctctggtgg agttatccct aatntttctg 420  
 ttctaacatt taagttgtat gcatcagtaa caggttcaaa agttatgtta ggttctcatt 480  
  
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 cttctaagaa aagtgtggat gtaactacaa atcaaaaagt aggacttagt caggatagca 1260  
 gtatggatta tatgggattg cttagaaagt caaaattaca tcagttcaag gaactnaacc 1320  
  
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 <400> 46  
  
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 aactcattnt tgctntgggc aaaatctgta aggaaaatgt atgcaagtga aaacctaat 180  
 ctaagcaaca ctgaaataag cagactgtta ggcaaaagntt ggaaagaaat gagtgaagnt 240  
  
 gagaaatntc cgnttatntca aagtgcnaag tntcttctgaa ctaagnttat acaagataat 300

ccaaattatc attacttctt taaaaaaagg aaaatcaatc aactaaataa tacaagtttt 360  
 tctaaaatgg ctccaatt aacaagtaat gatttaaatg catacaaaat ttttaaatat 420  
 ttaaacaatga gtgaaattat agaacacaaa gattingaaa gctttggcta ctttcaaagc 480  
 gtattcgag cttatcagaa cgaaaggaac ggcaatcaaa atacattttc gcctaaaatt 540  
 ctttcaaaaa gcattagtaa taacttcatt tcgaatgttc aattaaatgc taaatcaaat 600  
 aaaaataaaa gtgacagatt tgatgaaaat caacaaacgc acaaagaaac aactacgctg 660  
  
 cactactatt cagatgaagc acaacatagc tgtgaaaact ctttcaacat caataacgaa 720  
 gaagatttta atcaaaaata tcaataaaaa aaaagagaag atgttattga acttgactct 780  
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 <211> 834  
 <212> RNA  
 <213> Hydra magnipapillata  
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 ctaaccaaca ctgaaataag tagagtgcta gggaaagtct ggaaagaaat gagtgaagtt 240  
 gagaaattgc cgtttattca aagtgcaaag tgtcttcgaa ctaaattttt aatgattat 300  
 ccaaattatc aatacttgtg caaaaaacgg aaattcaatc aagtaataa tacaagtttt 360  
 tccaaaatgg cgtccaact aggaagtaat gatttaaatg catacgaat ttttaaatgt 420  
 ttaaacaatga gtgaaattat agaacacaaa gattingaaa gctttggcta ctttcaaagt 480  
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 aaaattaaaa gtgacagttt tgatgaaaat caacaaatgc aaaaagaaaa aactacgctg 660  
 cactactatt cagatgaaac acaacatttt aatcaaaatt ctttgacat aatagcgaa 720  
 gaagatttta atcaaaaata tcaataaaaa gaaagagaag atgttattga aattgactct 780  
 gaattaagag aattttttca gtctcttgag aaaggttttg gttattatga atga 834  
  
 <210> 48  
 <211> 1383  
 <212> RNA  
 <213> Hydra magnipapillata



<400> 48

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gataataaaa tgaagtgat ctttaatggt gttttactat tcgtggttgc acatggttta 180  
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<210> 49

<211> 694

<212> RNA

<213> Hydra magnipapillata

<400> 49

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 acacgattat tatatggcac aaaagtattt tgagcagctt aagtcataa ctaaagggtg 240  
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- <211> 560
- <212> RNA
- <213> Hydra magnipapillata
- <400> 50

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 ggtcaaagaa aaattaaagt aatttatgca aactatggta gaacctcgtc tcgtatatgc 240  
 tctgggaatt ttaatacga tttgccaag aatgtaaca atcaaaaaag atctctgaaa 300  
 gaagtgcgta acaagtgtc aggcaggtca tcttgcgttg ttcaagcatc gaatggagta 360

tttggcgacc catgttttgg aacgtataaa tatcttgaag tgcgttttta ttgtcaaag 420  
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- <211> 1246
- <212> RNA
- <213> Hydra magnipapillata
- <400> 51

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aagtcataca aaatatcgtt tgatttaaaa cccaagtcgt actcatatgg ttttcataat 240

gttattcaat tcaccgttgg tcattatatg agtaaatata gaaatagcac tccggcactt 300

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aatcctaaca gacaagtata tatcgatgaa cttccactca atgaatggac caaagttggt 420

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aatgtactta atgaagagaa caataaaccc caaaaatfff acaatgtcaa agtttttgtt 540

tcagctcctt tgcactatc tcataatggg ttaatacga atctaatct tgaaaatggg 600

gaaccaggtc aatcattaac gaatgctcta gagacaagag acaatgtgga taataatcac 660

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tttcgaatat gtatttttta tttaaaaata tatctttcaa atcgta 1246

**【심사관 직권보정사항】**

**【직권보정 1】**

**【보정항목】** 청구범위

**【보정세부항목】** 청구항 8

**【변경전】**

Hydra magnipalillata

**【변경후】**

Hydra magnipapillata

**【직권보정 2】**

**【보정항목】** 청구범위

**【보정세부항목】** 청구항 2

**【변경전】**

*Hydra magnipalillata*

**【변경후】**

*Hydra magnipapillata*