



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(54) Title: PESTICIDAL USE OF A PARASITIC FLAGELLATE FOR ELIMINATING OR SUPPRESSING HARMFUL ALGAE BLOOMS</p>		
<p>(57) Abstract</p> <p>The present invention relates to <i>Parvilucifera infectans</i>, a new parasitic organism described by Norén and Moestrup (in prep. "<i>Parvilucifera infectans</i>, gen. et. spec. nov. A parasitic dinoflagellate infecting thecate dinoflagellates.") capable of infecting and killing several toxic or potentially harmful dinoflagellates, method for infecting toxin producing dinoflagellates, and method for propagating <i>P. infectans</i>.</p>		

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**PESTICIDAL USE OF A PARASITIC FLAGELLATE FOR ELIMINATING OR SUPPRESSING HARMFUL ALGAE BLOOMS.**

**DESCRIPTION**

5 Technical field

The present invention relates to a parasitic microorganism being able to kill dinoflagellates, a method for eliminating or suppressing growth and/or replication of harmful dinoflagellates of, e.g., the genres *Dinophysis*, and *Alexandrium*, method for propagating said microorganism, and a composition containing said microorganism for carrying out said  
10 method.

The object of the present invention is to obtain a possibility of eliminating poisonous or otherwise harmful dinoflagellates from the sea, thereby avoiding accumulation of dinoflagellate related toxins in shellfish, such as mussels and oysters.

15

Background of the invention

Most coastal waters are even so often invaded by harmful microalgae blooms. Certain toxic algae will kill wild and farmed fish, and particularly the latter suffers, as it can not escape from the algae in contrast to the wild ones. This will cause an immediate economical impact  
20 on the breeders of farmed fish, as all stages of the farmed fish will be killed, and several years production will be affected. Other algae produce potent toxins that accumulate in filter-feeding shellfish and poison human consumers. Thus marketing and sale of such shellfish from such affected coastal areas is forbidden for long periods.

25 Bloom of microalgae is further harmful as the algae when dead will consume most of the oxygen present in the water, thereby causing bottom fauna death, and/or fish flight.

Toxic marine dinoflagellates can cause shellfish, e. g. mussels and oysters, to accumulate toxins in such concentrations that they become dangerous as human food. Toxic  
30 dinoflagellates can cause different types of shellfish poisoning ; DSP (Diarrhetic Shellfish Poisoning, caused by members of the genres *Dinophysis* and *Prorocentrum*), PSP (Paralytic Shellfish Poisoning, caused by the genres *Alexandrium*, *Gymnodinium*, and *Pyrodinium*) and NSP (Neurotoxic Shellfish Poisoning, caused by the genus *Gymnodinium*

*breve*). Those intoxications of the mussels inhibits the mussel industry as the mussels can not be harvested for shorter or longer periods. This production loss of the shellfish industry is estimated to 1-2 billions USD yearly. A further very important aspect is also that those who harvest shellfish for food consumption, have no possibility to check for high toxin  
5 content in the mussels. The shellfish industry has grown much during the last decades and is expected to grow even more due to the need of food for a growing human population. The aqua culture and shellfish market is expanding but is also suffering from marine pests, such as toxic marine phytoplankton.

10 A marine microalgae bloom is widely defined when the water is discoloured and/or comprises a cell concentration of  $1 \times 10^6$  cells per litre. The recording of those blooms is ancient but it is only in modern time we have been aware of the problems and suffer to a larger extent therefrom. It is also suspected that human eutrofication is causing the more intense and more frequently occurring blooms. The algae species that are producing toxins  
15 and are capable of forming blooms are mostly belonging to the dinoflagellate group of organisms.

In some Spanish fjords mussel harvesting sites have been closed down up to a half year. This could, hopefully be shortened down to some weeks, if a regulatory parasite to the toxic  
20 dinoflagellates is found.

Thus scientists are struggling to find methods to control those harmful microalgae blooms and are intensifying the efforts to find such methods (D.A. Anderson, Nature, **388**:513-514, August, 1997).

25 At present the best method is to spread huge amounts of clay into the water in order to clog the phytoplankton and hence rapidly precipitating them out of the water column (corresponding to the flocculation method in industrial sewage and waste water treatment). This is, however, a very costly method and labour intensive. The method has only been  
30 applied in the republic of China (D.A. Anderson, Nature, **388**:513-514, August, 1997).

JP patent specification 6001701 discloses red tide controlling material comprising a fibrous material carrier supporting a highly unsaturated fatty acid, whereby the material is supposed

to kill the red tide plankton in contact therewith.

JP patent specification 5169088 discloses the use of an attack bacteria of red tide plankton inhibitor comprising bentonite or kaolin-based clay-like particles.

5

JP patent specification 6016504 discloses a surfactant composition for controlling red tide, which surfactant composition comprises polyoxyalkylene alkyl ether, polyalkylene glycol fatty acid ester, polyoxyalkylene fatty acid amide, and polyoxyalkylene alkyl amine, which composition is sprayed onto the red tide plankton.

10

JP patent specification 8289693 discloses the use of radioactive compounds for killing red tide plankton.

F.J.R. Taylor, J. Fish. Res. Bd. Canada, **25**(10):2241-2245 (1968) discusses the parasitism of toxin-producing dinoflagellate *Gonyaulax catenella* by the endoparasitic dinoflagellate *Amoebophrya ceratii*, and concludes that it seems possible that the answer to harmful plankton blooms is the use of a biological control agent, similar to *A. ceratii*, as *A. ceratii* was not totally fatal to the host population in the case investigated.

20 Elbrächer, M. et al, in "Physiological Ecology of Harmful Algal Blooms", D.M. Anderson, A.D. Cembella & G.M. Hallegraeff, eds. Springer-Verlag Berlin Heidelberg, pp 351-363 (1998) discuss parasites of harmful algae as a tool for preventing harmful microalgal blooms. In this article the use of *A. ceratii* as proposed by Taylor, supra, seems to have been dismissed by Nishitani, L. et al. in "Toxic Dinoflagellates", D.M. Anderson et al, eds, Elsevier Sci. Publ. Co. New York, N.Y. pp 225-230 (1985).

Coats, D. W. et al., Aquat. Microb. Ecol., **11**:1-9, (1996) discuss parasitism of photosynthetic dinoflagellates in a shallow subestuary of Chesapeake Bay, USA. I.a., Coats et al discuss the parasitism of *A. ceratii* on *Alexandrium* (= *Gonyaulax*) *catenella* according to Taylor, supra, and the dismissal of Nishitani et al, supra, and are of the opinion that the Taylor's suggestion should be reexamined.

30 Scientists have been searching for a natural method of controlling these blooms and the use

of some kind of parasitic organism or predator has been in mind and requested, since parasites have been found to be an important regulating factor of a microalgae bloom. (Coats et al, supra). However, until today no such organism has been found that successfully is inhibiting a toxic, or otherwise harmful bloom, or could be industrially multiplied into an effective bloom inhibitor.

One parasite known to infect the toxic dinoflagellate genus *Dinophysis* is the parasitic dinoflagellate *Amoebophrya ceratii* (Taylor et al, supra). It is able to infect *Dinophysis* but is not able to graze down a bloom of the same species. (Coats et al, supra). This parasite has neither been actively used in attempts to control a dinoflagellate bloom, i. e. it has not been artificially cultured and reinserted in the natural environment as a pest controlling agent.

Thus the only in vivo method today, for terminating marine microalgae blooms is to put large amounts of clay into the sea to obtain a flocculation and precipitation of the algae. An intense research for biological controllers of the marine algae blooms is ongoing but today no such organism is known to work efficiently or is available to give an efficient control of the blooms. (Elbrächer et al, supra). The parasitic dinoflagellate *Amoebophrya ceratii* is able to infect *Dinophysis sp.* but is not able to control a bloom of the same species. *Amoebophrya ceratii* has neither been used to actively control a bloom of dinoflagellates.

20

#### Description of the present invention

It has, however, now surprisingly been found possible to solve this problem as a new parasitic organism has been found and isolated that is a lethal parasite to toxic and otherwise harmful dinoflagellates and more efficient than the previously known *Amoebophrya ceratii*. This new organism, hereinafter named *Parvilucifera infectans*, abbreviated *P. infectans*, is described by Norén and Moestrup (Norén, F. et. al. Europ. J. Protistol. **35**:233-254 (1999) "*Parvilucifera infectans* Norén et Mostrup gen. et. spec. nov. (Perkinsozoa phylum nov.): a Parasitic Flagellate Capable of Killing Toxic Microalgae").

FIG. 1 shows infection of *Parvilucifera infectans* in *Dinophysis*. Scale bars = 10 µm.

a. Early infection - 1 day in living specimen of *Dinophysis*. The arrow denotes *P. infectans*.

b. Late infection - 2 days, *Dinophysis* host is dead.

- c. Release of *P. infectans* sporangium from *Dinophysis*
- d. Papillar surface structure of *P. infectans*
- e. Restbody formation in sporangium of *P. infectans* after release of zooides
- f. Zooides of *P. infectans*.

5 FIG. 2 shows the taxonomic tree for *P. infectans*

*Parvilucifera infectans* has been deposited at the Culture Collection of Algae and Protozoa on the 4th day of December, 1998, under the deposition number CCAP No. 2060/1.

10 *P. infectans* is naturally, but very sparsely occurring at the Swedish west coast.

Berland, B.R. et al., Aquatic Microbial Ecology, 9:183-189 (1997) observed an organism that might have been *P. infectans*. However, the observation was lacking proper identification to provide for a guaranteed discovery. They were also explicitly stating that  
15 the organism found, was not a parasitic organism, but a sexual phase of *Dinophysis*. This has been proven to be false by Norén and Moestrup (supra) in their study of the organism.

The method to inhibit blooms of harmful plankton algae is straight forward: It is possible to maintain *P. infectans* in culture and to multiply it in large amounts. This can be scaled up in  
20 industrial production to yield large amounts of *P. infectans*. These cultured *P. infectans* are then spread over areas where a bloom of toxic dinoflagellates, susceptible for infection by *P. infectans*, occurs and causes a threat to human activities. *P. infectans* is used as an infection inoculum (a seed) and hence is speeding up an optional natural process within a much shorter time period than is the case in a natural process.

25

Until today no one has been able to culture the causative parasite or to determine the ecological parameters leading to a regulation of a phytoplankton bloom using parasites. By using *P. infectans* in waters having an ongoing harmful dinoflagellate bloom, consisting of dinoflagellates being susceptible to infection by *P. infectans*, the toxic species could be  
30 regulated in abundance and a non-toxic plankton community could be established in much shorter time period.

5 sporangia of *P. infectans*, are able to infect and kill a population of 20.000 cells of

*Dinophysis* sp. within three days and thereafter to produce a cascade of new parasites. This process has also been repeated with *Alexandrium fundyense*, *Alexandrium ostenfeldii*, *Alexandrium tamarense*, *Alexandrium anderssonii*, *Alexandrium catenella*, *Gymnodium sanguineum*, *Peridinium faeroensis* = *Pentaparsodinium dalei*, (all species in this document are named and described according to “Identifying Marine Phytoplankton”, Tomas C. R. Ed. 1997 Academic Press).

The life cycle of the *P. infectans* comprises, as far as known, of a zoide stage that is a small flagellated zoide (3.5 µm in length) which is infecting the host cell and thereafter develops into a sporangium stage having a diameter of 20 to 100 µm and comprising 500~ 2000 new zoides. During this latter stage the dinoflagellate host cell is gradually degraded and finally killed which takes between 1 to 2 days from infection. After the death of the host cell the new sporangium is released into the water and the sporangium is able to release the zoides and start a new infection cycle. The sporangium is also able to persist in a dormant stage (resting stage) and the survival of the resting stage is enhanced by lowering the temperature and to keep the cells out of the vicinity of potential host cells.

*P. infectans* is up to date known to infect members of the genres *Dinophysis*, *Alexandrium*, *Protoperidinium*, *Diplopsalis*, *Ceratium*, *Prorocentrum*, *Gymnodinium*, and *Gyrodinium*. *Dinophysis* comprises the toxic species *D. acuta*, *D. norvegica*, *D. dens*, and *D. acuminata* which all are infested and killed by *P. infectans*. *Alexandrium* comprises the toxic species *A. tamarense*, *A. ostenfeldii*, and *A. fundyense* which all are infected and killed by *P. infectans*. *Prorocentrum* comprises the toxic *Prorocentrum lima* and the bloom forming species *Prorocentrum micans* which both are infected and killed by *P. infectans*.

The method to inhibit blooms of harmful microalgae comprises at least two parts, viz

- i. the method of culturing *P. infectans* to yield pure large quantities of *P. infectans*, and
- ii. the method of adding *P. infectans* into marine and limnic environments to enhance the extinction of a harmful microalgae bloom or a microalgae bloom that is causing nuisance.

30

#### Propagation

A. Sporangia of *P. infectans* are added to a monoculture of dinoflagellates susceptible to infection by *P. infectans*. The dinoflagellate culture should be maintained at optimal



condition for the specific species as specified by culture collecting centres, and by common practise known to the one skilled in the art. The temperature should exceed 15° C in order to obtain a rapid infection.

5 Three alternatives to collect *P. infectans* are thereby possible:

i. Have a running culture of dinoflagellates, adding continuously new cultured dinoflagellates into the vessel comprising *P. infectans* and collecting sporangia, and/or infected dinoflagellates continuously from the bottom of the culturing vessel.

10

ii. Have a batch culture of dinoflagellates, susceptible to infestation by *P. infectans*, and to add *P. infectans*. When the infection is completed the bottom water of the vessel is collected where all of the new produced *P. infectans* will be present.

15 iii. Taking natural water from the microalgal blooming area into a batch culture and add *P. infectans* thereto. After the batch has been extensively infected with *P. infectans*, the water is returned to the bloom area as an infection inoculum.

20 After treatment i. or ii. the collected *P. infectans* is placed dark and in low temperature, +5°C to +8°C. *P. infectans* will go into a dormitory stage and optional living specimens of the host dinoflagellate will die. When a pure *P. infectans* population is obtained, antibacterial action can be taken as a treatment of the water with an antibiotic or other antibacterial agents.

25 The final product, i.e. a water having a high concentration of *P. infectans* can be stored for long time at +5°C and in darkness. Hereby, the water can be cryopreserved in order to store the *P. infectans* before use. *P. infectans* can also be stored by dry-preservation.

30 B. The use of culturing media to culture *P. infectans*. The closest phylogenetical relative is the family Perkinsidae (phylum apicomplexa) which can be cultured using fluid thioglycollate medium.

II. The aqueous solution comprising the *P. infectans* sporangium is spread over a water

containing a dinoflagellate of the above genus, e.g.; by spraying from an aircraft, spraying using a water canon from a boat, or distribution from a long tube comprising evenly distributed nozzles, which tube is arranged perpendicular to the longitudinal axis of a boat.

- 5 According to the present invention a concentration of 1000 sporangia comprising at least 1000 zooids each (c.f. above) per litre of an aqueous medium will kill 1,000,000 dinoflagellates per litre within three days. However, in order to obtain a rapid and efficient killing effect, 1,000,000 parasites per litre of aqueous medium seems to be more appropriate. The cascade effect caused by the short generation time of the parasite and the
- 10 high reproduction rate is strongly enhancing the killing effect within one week so that the major part of the microalgae bloom is killed successively.

#### Experimental results

- The killing and/or suppressing effect of *P. infectans* has been verified in a number of
- 15 experiments (>20), which all show that the results obtained are reproducible. At all experiments control samples were run simultaneously with samples that were non-infected. The degree of lethal infection after one week incubation was between 90 % and 100 % for the *Dinophysis* species *D. acuta*, *D. norvegica*, and *D. acuminata*, and the *Alexandrium* species *A. fundyense*, *A. ostensfeldii*, *A. tamareense*, *A. anderssonii*, *A. catenella*,
- 20 *Gymnodinium sanguineum*, *Peridinium faeroensis* = *Pentapharsodinium dalei*. Following species have been found to be infected occasionally in natural samples infected by *P. infectans*, viz: *Dinophysis acuta*, *Dinophysis norvegica*, *Dinophysis acuminata*, *Dinophysis caudata*, *Dinophysis fortii*, *Dinophysis miles*, *Dinophysis mitra*, *Dinophysis rotundata*, *Dinophysis sacculus*, *Dinophysis tripos*, *Alexandrium fundyense*, *Alexandrium ostensfeldii*,
- 25 *Alexandrium tamareense*, *Alexandrium acatenella*, *Alexandrium catenella*, *Alexandrium angustitabulatum*, *Alexandrium cohorticula*, *Alexandrium hiranoi*, *Alexandrium minutum*, *Alexandrium monilatum*, *Alexandrium tamiyavanichi*, *Pyrodinium bahamense*, *Pyrodinium bahamense var. compressum*, *Gambierdiscus toxicus*, *Ostreopsis lenticularis*, *Ostreopsis siamensis*, *Ceratium furca*, *Ceratium tripos*, *Ceratium fusus*, *Ceratium macroceros*,
- 30 *Ceratium* sp., *Diplopsalis* sp., *Proto-peridinium crassipes*, *Proto-peridinium brevipes*, *Proto-peridinium curtipes*, *Proto-peridinium depressum*, *Proto-peridinium bipes*, *Proto-peridinium* sp., *Prorocentrum micans*, *Prorocentrum lima*, *Prorocentrum concavum*, *Prorocentrum mexicanum*, *Prorocentrum minimum*, *Prorocentrum* sp., *Gymnodinium breve*,

*Gymnodinium mikimotoi*, *Gymnodinium catenatum*, *Gymnodinium* sp., *Peridinium polonicum*, *Pfiesteria piscicida* and *Gyrodinium* sp.

According to the experimental results a wide range of thecate dinoflagellates is infected  
5 by *Parvilucifera infectans* and it is thereby concluded that other related species not present  
in those investigations are infected by *P. infectans*, as well, such as the tropic and temperate  
variants and species.

**CLAIMS**

1. Isolated *Parvilucifera infectans*, a new parasitic organism deposited at CCAP under deposition number CCAP 2060/1 and described by Norén and Moestrup (Norén, F. et al, Europ. J. Protistol. **35**:233-254 (1999) "*Parvilucifera infectans*, Norén et Moestrup gen. et. spec. nov. (Perkinsozoa phylum nov.): a Parasitic Flagellate Capable of Killing Toxic Microalgae.
- 5
2. Method for eliminating or suppressing algal bloom caused by thecate dinoflagellates wherein *P. infectans* is distributed in a water comprising one or more of said algae.
- 10
3. Method according to claim 2, wherein the algae are selected from the group comprising the following species: *Dinophysis acuta*, *Dinophysis norvegica*, *Dinophysis acuminata*, *Dinophysis caudata*, *Dinophysis fortii*, *Dinophysis miles*, *Dinophysis mitra*, *Dinophysis rotundata*, *Dinophysis sacculus*, *Dinophysis tripos*, *Alexandrium fundyense*, *Alexandrium ostenfeldii*, *Alexandrium tamarense*, *Alexandrium acatenella*, *Alexandrium catenella*, *Alexandrium angustitabulatum*, *Alexandrium cohorticula*, *Alexandrium hiranoi*, *Alexandrium minutum*, *Alexandrium monilatum*, *Alexandrium tamiyavanichi*, *Pyrodinium bahamense*, *Pyrodinium bahamense* var. *compressum*, *Gambierdiscus toxicus*, *Ostreopsis lenticularis*, *Ostreopsis siamensis*, *Ceratium furca*, *Ceratium tripos*, *Ceratium fusus*, *Ceratium macroceros*, *Ceratium* sp., *Diplopsalis* sp., *Protoperidinium crassipes*, *Protoperidinium brevipes*, *Protoperidinium curtipes*, *Protoperidinium depressum*, *Protoperidinium bipes*, *Protoperidinium* sp., *Prorocentrum micans*, *Prorocentrum lima*, *Prorocentrum concavum*, *Prorocentrum mexicanum*, *Prorocentrum minimum*, *Prorocentrum* sp., *Gymnodinium breve*, *Gymnodinium mikimotoi*, *Gymnodinium catenatum*, *Gymnodinium* sp., *Peridinium polonicum*, *Pfiesteria piscicida* and *Gyrodinium* sp.
- 15
- 20
- 25
- 30
4. Method according to claim 2, wherein an aqueous solution comprising *P. infectans* sporangia are distributed, whereby the concentration of sporangium in said solution is at least 1000 sporangia per litre.
5. Method according to claim 2, wherein the *P. infectans* is present in a sporangium stage.
6. Method according to claim 2, wherein the *P. infectans* is present in a zoide stage.

7. Method for propagating *P. infectans*, **characterized** in that *P. infectans* is cultured in a medium containing thioglycollate.
- 5 8. Method for propagating *P. infectans*, **characterized** in that *P. infectans* are added to a monoculture of a dinoflagellate being susceptible to infection by *P. infectans*, maintaining the dinoflagellate culture at optimum conditions above 15°C, collecting dead and infected dinoflagellates, and storing said infected material in an aqueous solution or optionally subjecting it to lyophilisation.
- 10 9. Method according to claim 8, wherein the *P. infectans* is stored at +5°C and in darkness.
10. Method for propagating *P. infectans*, **characterized** in that *P. infectans* are added to water containing dinoflagellates susceptible to infection by *P. infectans*, maintaining the  
15 dinoflagellate culture at optimum conditions above 15°C, collecting dead and infected dinoflagellates, and storing said infected material in an aqueous solution.

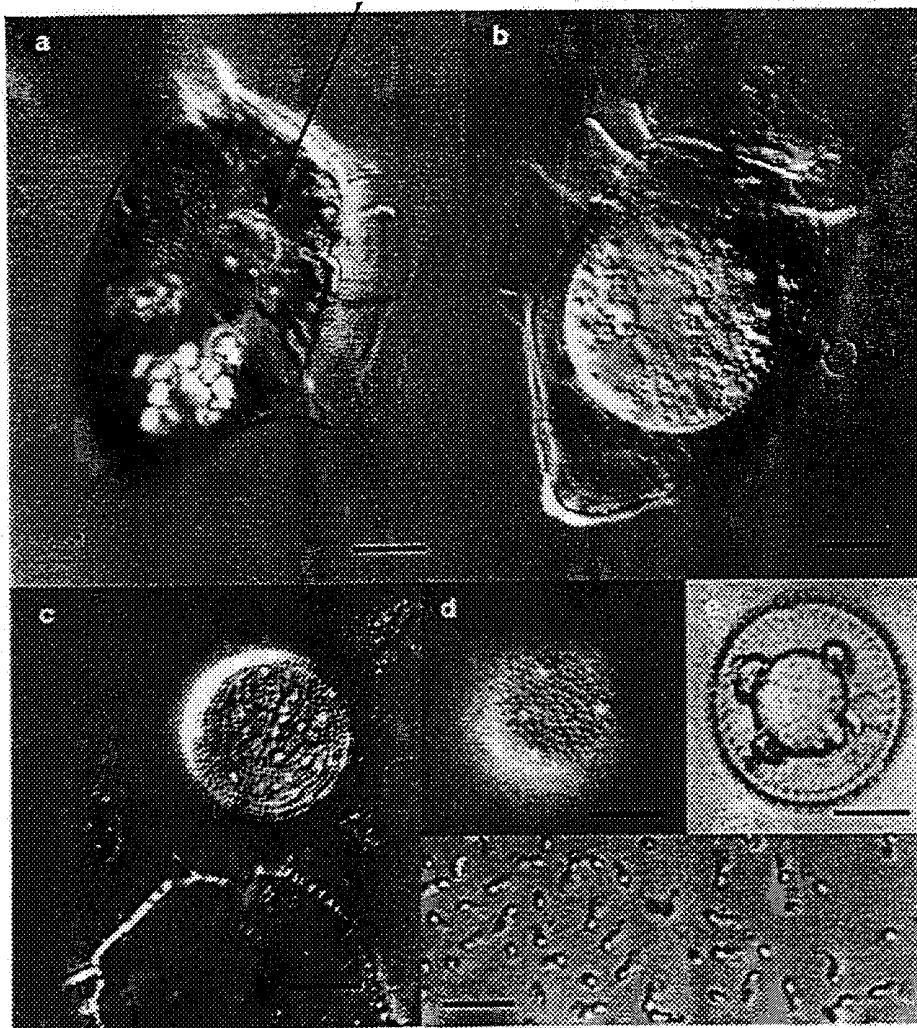


FIG. 1

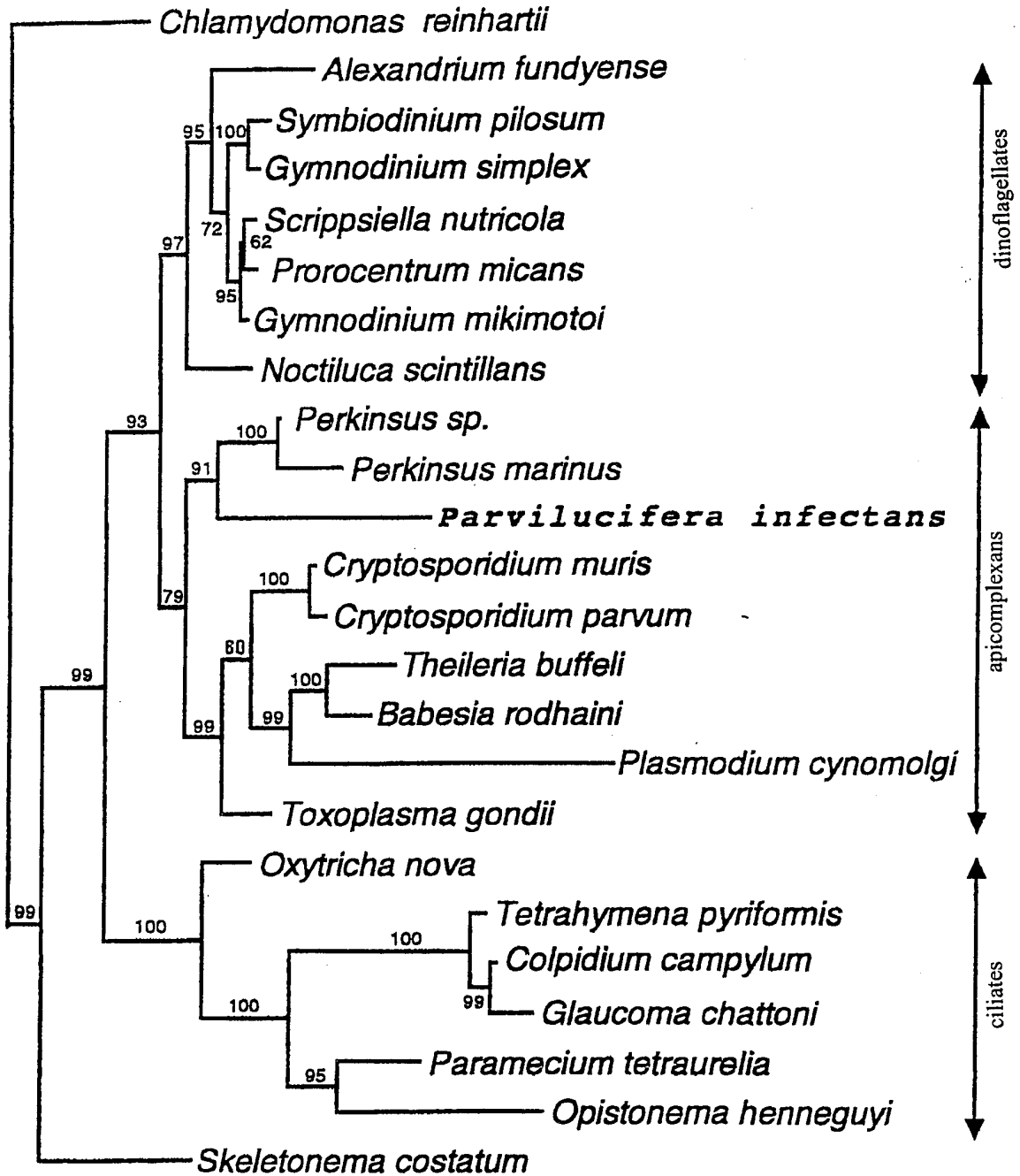


FIG. 2

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/00170

## A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A01N 63/00, C12N 1/10 // (C12N 1/10, C12R 1:90)  
 According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A01N, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Europ. J. Protistol., Volume 35, 1999, Fredrik Norén et al, "Parvilucifera infectans Norén et Moestrup gen. et sp. nov. (Perkinsozoa phylum nov.): a Parasitic Flagellate Capable of Killing Toxic Microalgae" page 233 - page 254  --	1-10
X	Aquatic microbial ecology, Volume 11, August 1996, D. W. Coats et al, "Parasitism of photosynthetic dinoflagellates in a shallow subestuary of Chesapeake Bay, USA" page 1 - page 9  -- -----	1-10

 Further documents are listed in the continuation of Box C.

 See patent family annex.

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