Molecular Identification of the Toxic
*Alexandrium tamiyavanichii* (Dinophyceae) by
the Whole-cell FISH Method

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The dinoflagellate *Alexandrium tamiyavanichii* Balech, a producer of toxins causing paralytic shellfish poisoning (PSP), has recently been considered as one of main organisms responsible for toxification of shellfish in Japan. In this study, *A. tamiyavanichii* was subjected to a molecular phylogenetic analysis inferred from 28S rDNA D1-D2 sequences and a species-specific LSU rRNA-targeted oligonucleotide DNA probe was designed to identify *A. tamiyavanichii* using the whole-cell-FISH (fluorescence *in situ* hybridization). The sequences of the 28S rDNA D1-D2 region of *A. tamiyavanichii* showed no difference from *A. cohorticular* AF174614 (present name *A. tamiyavanichii*) and formed a distinct clade from the *tamarensis* species complex. The probe, TAMID2, reacted specifically with *A. tamiyavanichii* cultured cells, without any cross-reaction with other species belonging to the same genus, including *A. tamarensis*, *A. catenella*, *A. affine*, *A. fraterculus*, *A. insuetum* and *A. pseudogonyaulax*. In a test of cross-reactivity with a field sample, TAMID2 reacted consistently with only *A. tamiyavanichii*, indicating that the present protocol involving the TAMID2 probe might be useful for detecting toxic *A. tamiyavanichii* in a simple and rapid manner.

Key words: Whole-cell-FISH (fluorescence *in situ* hybridization), DNA probe, *Alexandrium tamiyavanichii*

**Introduction**

Recently, the morphologically and molecular-phylogenetically poorly-defined toxic species *A. tamiyavanichii* has caused PSP toxification of shellfish. Since its first confirmation in 1999 in Harima Nada, Japan, PSP toxification by this species has spread, and consequently caused economic and sanitary problems (Hashimoto et al., 2002).

*A. tamiyavanichii* (*Protogonyaulax tamiyavanichii*) found in the Gulf of Thailand as a toxic strain was reported by Kodama et al. (1988) and Hashimoto et al. (2002) demonstrated that wild vegetative cells contained GTX1-5, STX and C1-2 and bivalves feeding on this alga were intoxicated with GTX1-5, STX, (deSTX), (neoSTX), and C1, 2, (3), (4), showing strong toxicity and diversity of toxin components.

Difficulties in field monitoring for *A. tamiyavanichii* are derived not only by its long-chain-forming features shared by *A. fraterculus* (Balech) and *A. affine* (Inoue and Fukuyo), but also from the overlapping occurrence of their vegetative cells in the same water column. In particular, the introduction of the toxic *Alexandrium* species into a water column with low cell density is likely to be beyond the limits of traditional monitoring methods. Therefore, a rapid and reliable identification method for the toxic *Alexandrium* is required.

DNA probing using fluorescent-labeling has been used recently as an alternative to conventional identification methods; targeted probing to either rRNA
or rDNA has been given much attention as a powerful culture-independent tool (Giovannoni et al., 1988; Amann et al., 1990; DeLong and Shah, 1990; Distel et al., 1991; Worden et al., 2000). Despite the extensive literature on *Alexandrium* and other species causing harmful algal blooms (HABs), the application of DNA probing for species identification and detection is restricted to a few reports (Adachi et al., 1996a; Scholin et al., 1996; Miller and Scholin et al., 1996; Parson et al., 1999). Development of reliable molecular biological identification methods is a prerequisite for tackling the problems of toxic *Alexandrium*.

This study described the morphological fine features of *A. tamiiyavanichii* isolated in Japan and constructed the molecular phylogenetic relationships of the species with long chain-forming *A. affine* and *A. fraterculus* inferred from sequences of the 28S rDNA D1-D2 domain. Also, we designed a species-specific oligonucleotides DNA probe for identification and detection of the species based on the whole-cell FISH (fluorescence in situ hybridization) method.

### Materials and Methods

#### Cultures

Clonal cultures of seven *Alexandrium* species, *A. affine*, *A. catenella*, *A. fraterculus*, *A. insuetum*, *A. pseudogonyaulax*, *A. tamarensis* and *A. tamiiyavanichii*, and other dinoflagellates, *Prorocentrum micans*, *P. minimum*, *Heterocapsa circularisquama*, *H. triquetra*, *Gymnodinium catenatum*, *Chattonella marina* and *Heterosigma akashiwo*, were established from an isolated vegetative cell in natural sea waters (Table 1). All the isolates were incubated in a SW II medium (Sako et al., 1990) at 20°C, on a 14:10 L:D regime at 100 μEm⁻²S⁻¹ using cool white bulbs. Morphological taxonomy followed the criteria of Fukuyo (1985).

#### Molecular phylogenetic analysis

Three isolates of *A. tamiiyavanichii* and one isolate of each of *A. affine* and *A. fraterculus* listed in Table 1 were subjected to sequence-determination of the 28S rDNA D1-D2 domain. DNA preparation and sequencing procedures were conducted according to

<table>
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<tr>
<th>Species</th>
<th>Strain</th>
<th>Origin</th>
<th>Toxicity</th>
<th>Probe reactivity</th>
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<td>ULW9903</td>
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<td><em>Heterosigma akashiwo</em></td>
<td>OS11</td>
<td>Osaka Bay, Japan</td>
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Note: PSP toxicity was determined by C.J. Kim (unpublished data); Yoshida et al., 2001; Sako et al., 1999 and S.A. Yoshimatsu (personal communication), respectively.

⁴The isolates labeled with the probe TAMID2.

⁵The isolates not labeled with the probe TAMID2.
the methods described by Adachi et al. (1996b). Approximately 700 nt of the LSU rDNA D1-D2 was amplified by PCR using the primers designed by Scholin et al. (1994). PCR amplification was performed by denaturing at 96°C for 3 min as an initial step, followed by 30 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The Final extension was performed at 72°C for 7 min. The positions and sequences of the primers in1F and in1R in the 5′-3′ direction for sequencing of each species were as follows: 170-190, AAGAAAGTGCTAATGAGGT and 497-478, CAATAACTGAGCAGCAG for *A. fraterculus*, 183-202, TGAGGTTGAGAATCCTGTTT and 596-577, GCACACACACATGATACCA for *A. affine*, and 171-152, GCTGCGAAGAACAGAAATC and 488-467, GCTGCGACGACAGAAGAATC for *A. tamiyananicii*. Sequence data obtained in this study and retrieved from GenBank (accession numbers shown in Fig. 2) were used for phylogenetic analysis using ClustalX (Thomson et al., 1997). The alignment generated was revised manually to increase alignment similarity, and converted to a PHYLIP format. Phylogenetic analyses were carried out using the PHYLIP package (Felsenstein, 1995). Bootstrapping confidences (Felsenstein, 1985) with 100 replications were generated from SEQBOOT, and a distance matrix was calculated with DNADIST with 100 multiplex data sets using the F84 distance model (Felsenstein, 1984). Phylogenetic trees were constructed using a neighborjoining (NJ) option with 100 multiple data (Saitou and Nei, 1987). A transition/transversion ratio of 2.0 was selected and data sets of 100 were conducted using empirical base frequencies with one category of substitution rates. CONSENSE of PHYLIP and TREE VIEW (Page, 1996) were used for the consensus tree and tree reconstruction, respectively.

**DNA probe design**

The 28S rDNA D1-D2 sequences of *Alexandrium* from GenBank and this study were aligned to screen species-specific regions for designing an *A. tamiyananicii*-specific DNA probe. Oligonucleotide DNA probe TAMID2 specific for *A. tamiyananicii*, composed of 23 sequences (445-5'TGTACCTAGGACACAACCACATA3'-426, complementary to coding strand), was designed. The 5′ end of the sequence was labeled with FITC (fluorescein isothiocyanate) to visualize the hybridized intact cells under an epifluorescence microscope. The fluorescent DNA probe was synthesized commercially and purified through a cartridge (Amersham Pharmacia Biotech, Tokyo, Japan).

**Screening of the optimum hybridization and washing temperatures**

To determine the optimum hybridization and washing temperatures, TAMID2 was tested with combinations of five hybridization temperatures from 35 to 55°C and seven washing temperatures from 35 to 65°C using *A. tamiyananicii* (TAMI2201) cultured cells. Hybridized cells were counted with a Coulter EPICS Elite Flow Cytometer (Coulter) equipped with a single 6-W argon ion laser (excitation, 488 nm of 250 Mw). FITC was detected at 520-530 nm with FS (forward scatter) and SS (side scatter) as parameters. Detection sensitivity of the flow cytometer was calibrated with fluorescently labeled beads (Flow-Check™ Fluorospheres, Backman Coulter).

The DNA probe TAMID2 showed the highest relative fluorescent intensity with a hybridization and washing temperature of 35°C and the second highest with hybridization at 35°C and washing at 40°C (data not shown). To raise probe specificity, we adapted the second best hybridization: washing temperature set at 35:40°C for this whole cellFISH method.

**Labeling-reactivity and specificity using whole-cell FISH**

To test the reactivity and specificity of labeling by the DNA probe TAMID2, cultured cells of the *Alexandrium* species and representative red-tide forming dinoflagellates (see Table 1) were harvested in the exponential growth phase. Pelleted cells were dehydrated with an ethanol series of 50% for 1 min and 99% for 30 min at room temperature with still state to ease for DNA probes to hybridize and to remove autofluorescence from chlorophylls without fixation procedure. The dehydrated cells were air-dried completely, then subjected to hybridization by adding 200 μL of a hybridization buffer (40% formamide/5×SSC; 83 mM sodium nitrate and 83 mM sodium citrate, pH 7.0) containing the DNA probe (denatured at 100°C for 5 min, chilled on ice and adjusted to a final concentration of 0.5 pmol). Cells were hybridized with the fluorescence probe at 35°C for 5 min in a water bath. Hybridized cells were incubated in a 5×SSC washing buffer at 40°C for 5 min twice to remove the remaining unbound DNA probes and non-specifically hybridized probes. The remaining 10-20 μL of buffer containing the cell pellet
was gently pipetted, moved onto the slidegalss and observed under an epifluorescent microscope (Nikon ECLIPSE E800) equipped with a fluorescein band pass filter set for FITC and counter stain (TRITC. PI) (excitation 450-490 nm; DM, 505 nm; BA, 520 nm) at 400 times magnification. Fluorescence and light images of cells were taken using a color chilled 3 CCD camera (Hamamatsu C5810) with an exposure time of 0.4 sec.

To test the cross-reactivity of the DNA probe, natural sea water samples were collected by netting (20 μm-pore size) on August 29, 2002 in Maizuru Bay, Kyoto, Japan. Concentrated samples were prepared in polypropylene bottles without fixation, maintained in an icebox (ca. 5-10°C) during transport to the laboratory and subjected to the whole-cell FISH within 5 h after sampling. In the laboratory, field samples were concentrated further with a sieve (20 μm-pore size) and placed in 50-mL polypropylene disposable centrifuge tubes at room temperature. One mL of cultured A. tamiyavanichii cells (ca. 1,000 cells/mL) was added to 1 mL of the natural seawater samples to test cross-reactivity of the DNA probe. Hybridization and washing temperatures were at 35-40°C. After treatment, the cells were resuspended and observed as described above.

Results

Morphological description of A. tamiyavanichii

The diameter of the A. tamiyavanichii vegetative cell (Fig. 1) ranged from 40 to 60 μm with a slightly slender shape. As expected, the cells formed long chains (Fig. 1A). The apical pore complex (APC) had a relatively large apical attachment pore (a.a.p.) (Fig. 1D) and connected directly to the 1' plate inscribed with a ventral pore (v.p.) (Fig. 1C). The anterior sulcal plate (s.a.) (Fig. 1B) assumed a unique form with a precingular part (p.pr.) (Fig. 1E) with a dome-like shape, which overlapped the bottom angle of 1' and unciform apophysis (a.u.) of the hooked end and chopped posterior sinus (s.) were characterized in Fig. 1E. The posterior attachment pore (s.p.) was nearly central in the posterior plate (Fig. 1F).

Molecular phylogenetic relationships of A. tamiyavanichii within the genus Alexandrium

Sequences of the 28S rDNA D1-D2 region of three A. tamiyavanichii strains, TAM12201, TAM12207 and TAM122012, were 710 nt in length without insertion or deletion. A. affine (AFF37) and A. fraterculus (DPW9709) displayed sequences of 711 and 717 nt in length. Sequences of Alexandrium determined in

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Fig. 1. Morphological designation of A. tamiyavanichii: A, figure showing long chain-forming vegetative cells; B, ventral view; C, apical view; D, structure of apical pore complex; E, ventral view showing anterior sulcal plate (s.a.) with a precingular part (p.pr.); F, antapical view. Scale bar=30 μm. Abbreviations by Balech (1995) and Yoshida (2000) were used.
the present study (A. affine AFF37, AB088227; A. fraterculus DPW9709, AB088244; A. tamiyavanichii TAM12201, AB088264; A. tamiyavanichii TAM12207, AB088265; A. tamiyavanichii TAM122012, AB088263) and retrieved from GenBank (see Fig. 2) were subjected to a molecular phylogenetic analysis. Fig. 2 shows the molecular phylogenetic relationships inferred from the sequences of 28S rDNA D1-D2 region of the genus Alexandrium. A. tamiyavanichii strain TAM12201 and A. cohorticula AF174614 showed no differences in sequence, suggesting that they are synonymous. The A. tamiyavanichii clade is supposed to have diverged prior to the ‘tamarensis species complex’ and latter than the basal clades composed of A. anderssonii, A. margalefii, A. ostenfeldii, A. minutum and A. insuetum (Fig. 2). The A. tamiyavanichii clade had relatively close sister relationships with A. fraterculus as well as A. affine, but these two clades were clearly distinct from each other.

**TAMID2 reactivity to targeted A. tamiyavanichii cells and its cross-reactivity to non-targeted Alexandrium and other dinoflagellates**

The sequence of the TAMID2 had many nucleotide mismatches with the corresponding region in A. affine, A. anderssonii, A. catenella, A. excavatum, A. fraterculus, A. insuetum, A. margalefii, A. minutum, A. ostenfeldii and A. tamarensis as shown in Table 2.

Fig. 3 shows the reactivity and specificity of the oligonucleotide probe TAMID2 to targeted A. tamiyavanichii and non-targeted toxic A. tamarensis, A. catenella and non-toxic A. affine and A. fraterculus cultured cells. TAMID2 reacted with A. tamiyavanichii (TAM12201) cultured cells in the whole-cell FISH, and most cells were clearly visualized with strong fluorescent intensity against a dark background. A. tamiyavanichii cultured cells subjected to the whole-cell FISH without the probe TAMID2 showed only pyrenoid-like spots (Fig. 3 B and B’). The probe showed no traces of cross-reaction with various other Alexandrium species, including toxic A. tamarensis and A. catenella, and non-toxic A. affine, A. fraterculus, A. insuetum and A. pseudogonyaulax or with cultured cells of other genera, Prorocentrum micans, Prorocentrum minimum, Heterocapsa circularisquama, Heterocapsa triquetra, Gymnodinium catenatum, Chattonella marina and Heterosigma akashiwo (Table 1). Autofluorescence in photosynthetic organisms, which has become a major problem in whole-cell FISH, was completely eliminated by ethanol dehydration in all species tested in the present study, therefore, it did not act as an obstacle to effective hybridization.

**Detection of the targeted A. tamiyavanichii cultured cells and cross-reactivity in natural field samples**

The DNA probe TAMID2 reacted with only A. tamiyavanichii cultured cells without any cross-reactivity to other components of a natural field sample composed of various diatoms, dinoflagellates and zooplanktons as shown in Fig. 4. The fluorescent intensity of A. tamiyavanichii cultured cells was strong, and each cell was completely covered with the signal. Nonspecific staining of detritus-like amorphous clusters was occasionally found in low frequencies but could be removed by size-fraction of filtration. Autofluorescence in the field sample was not completely eliminated, as typified by that

Fig. 2. Putative molecular phylogenetic tree inferred from 28S rDNA D1-D2 sequences of the genus Alexandrium. The tree topology was derived by the N-J method using ca. 570 nt. Bootstrap values over 60% are shown at the internodes. Alexandrium affine, A. fraterculus and A. tamiyavanichii isolates sequenced in the present study are boxed and the sequence data of their 28S rDNA D1-D2 regions have been deposited in the EMBL/DDBJ/GenBank. Gonyaulax spinifera was used to root as an outgroup taxon.
Table 2. *Alexandrium* species-specific region of LSU rDNA D1-D2 domain

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<th>Account no.</th>
</tr>
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</tr>
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<td>A. andersonii</td>
<td>GCA----------ACTTGGGCGGCA</td>
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<td>A. catenella</td>
<td>TGGGTTTGG--CGCAATGGTCA</td>
<td>AF318220</td>
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<td>A. fraterculus</td>
<td>TCGATGTGAA---TTGCAAAATGCA</td>
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<td>A. insuetum</td>
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</table>

*Sequences of A. affine (AFF37), A. fraterculus (DPW9709) and A. tamiyavanichii (TAMI2201) were determined in the present study.*

*Sequences are listed 5’ to 3’ and the sites are from 426 to 441 (see Scholin et al., 1994).*

Fig. 3. Epifluorescence microscopic (A to F) and light microscopic images (A’ to F’) of cultured *Alexandrium* cells hybridized with DNA probe TAMID2 or treated without the probe (B and B’): A and A’ and B and B’, A. tamiyavanichii (TAMI2201); C and C’, A. tamarensis (KJC9711); D and D’, A. catenella (DPC8); E and E’, A. affine (AFF37); F and F’, A. fraterculus (DPW9709). Scale bars=30 μm.

Fig. 4. Epifluorescence microscopic (A) and light microscopic images (A') of concentrated natural seawater sample hybridized with TAMID2. Arrows indicate targeted A. tamiyavanichii (TAMI2201) cells and scale bar=30 μm.

of *Dinophysis* spp., but its red color was clearly distinguished from the green of FITC with which the DNA probe was labeled.

Discussion

The DNA probing methods for field organisms should be simple and rapid, especially if the target organisms are near death and decomposition of their rDNA and rRNA. The DNA probe sequences should be designed with high specificity to distinguish the target organism not only from the related species but also from a great number of other bio-communities in the field.

In this FISH protocol, we omitted the CTAB (cetyltrimethyl ammonium bromide) process usually used for puncturing the cell wall. Still, the DNA probes were well hybridized with rRNA in the cytoplasm of most intact *A. tamiyavanichii* cells. Formamide was used at the concentration of 40% in a hybridization buffer of 5 X SSC, though there was a report that high concentration of formamide (>20%)
inhibited the whole cell hybridization (Dedysh et al., 2001). We also skipped the step of cell fixation, based on our empirical observation that most cellular rRNAs exposed to glutaraldehyde leaked from cells, during transportation of a field sample to the laboratory at a low temperature of 5-10°C. A similar phenomenon was pointed out by Scholin et al. (1996), who reported that Pseudo-nitzschia species often exuded their cell contents when exposed to formalin and other aldehyde-type preservatives. To prevent cell degradation during preservation, they recommended the use of an ethanol/saline-based solution for fixation-preservation. Worden et al. (2000) compared the effect of the fixation agents glutaraldehyde and paraformaldehyde on labeling intensity, and stated that glutaraldehyde (0.1% final concentration) caused higher fluorescence background and lower signal strength in cultured Prochlorococcus than a paraformaldehyde fixation. A possible explanation for this is that fixation may cause cell contraction or expansion, resulting in damage to the cell wall or even the nuclear membrane and consequent leakage of cell components. Such damage would be accelerated by agitating the natural seawater samples during the transportation. Damage was more remarkable when relatively high concentration of fixatives, ethanol and acetone, were added to the samples. We know empirically that nucleic acid is decomposed during the cell fixation with aldehyde-like agents. Therefore, it is considered that field samples should be concentrated on a filter, and then treated with a series of ethanol solution to remove autofluorescence and effect dehydration. Filtered dry samples should be transported. These treatments can prevent the collapse and loss of cell, which are significant obstacles in quantitative enumeration. Additionally, temperature control is essential to accomplish a stable hybridization system.

Whole-cell hybridization by the simple method described above showed that A. tamiavanichii cells were thoroughly labeled with TAMID2, indicating that the DNA probe reacted with the targeted rRNAs with good access to the target sites, and that the target region harboring significant substitutions between species is a relatively plane structure without folding, helices or binding with other molecules. Only the nuclear part of the cells did not permit the probe to label, ensuring an unlabeled part remained.

We chose an oligonucleotide in the variable domain D2 of 28S rDNA harboring an Alexandrium species-specific region. The DNA probe TAMID2 designed for A. tamiavanichii did not cross-react with any other Alexandrium species tested in this study. It also showed no cross-reactivity with field sample, probably because of the high sequence-specificity of TAMID2 and the use of the rapid labeling method without CTAB treatment, though DNA probe capture by detritus aggregates and mucous materials was sometimes observed. For easy permeabilization of cell wall for binding of the target molecules in cytosol and the DNA probe molecules, several agents such as paraformaldehyde, diethyl ether, xylene (Arnoldi et al., 1992), hydrochloric acid, (Macnaughton et al., 1994), lipase (Davenport et al., 2000) and lysozyme (Schwiertz et al., 2000) has been compared, but even the use of only formamide of 40% in this study did not require further agents searching to obtain more effective labeling.

Direct cell counting was also conducted to assess quantification as the ratio of cell counts under epifluorescent and light microscope. The ratio was over 99% during the exponential growth phase and fell below 80% from the stationary growth phase, demonstrating that dead and collapsed cells were not available for probing. This confirms that labeling-intensity was predominantly dependent on the physical condition of cells. We observed, however, that A. tamarensie vegetative cells from a field sample were of various sizes, implying perhaps different physical status, but labeling-intensity was very high and even (Kim, unpublished data).

Hybridization and washing temperatures for labeling with TAMID2 were positive at 35:35, 35:40, 35:45, 40:40, 40:45, and 45°C:45°C, while temperatures of over 50°C were not appropriate for either process because probes were denatured during both processes. Among the regimes tested, 35:35 and 35:45 were the best, illustrating that DNA probes were well hybridized under the condition when both hybridization and washing temperatures were approximately 10-15°C and 10°C lower, respectively than melting temperature (Tm) (TAMID2, 52.7°C), which is defined as the equilibrium temperature at which half of the probe-target duplexes are dissociated (Stahl and Amann, 1991). To raise probe specificity to field samples, hybridization and washing temperatures should be set at least 5°C lower than Tm.

Fluorescent signal intensity was generally strong during the exponential growth phase. However, it declined steeply with the degradation of cells. Another drawback arose from the fast fading of the fluores-
cence signal. In order to not only diminish the fading, but also enhance the fluorescence signal, treatment of antifade agents and comparison of various fluorescence agents such as indocarbocyanine (Cy3), indodicylcarbocyanine (Cy5), rhodamine, Texas red, 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUO-S), Oregon green 538, and testing of multiple probes are necessary.

Further studies should be conducted to facilitate cell detection and quantification. We expect improvements to be achieved shortly through development of a simple system for filtration and hybridization of field samples.

Acknowledgements

The authors thank Drs. S. Yoshimatsu and M. Yamaguchi for providing algal strains. This work was supported in part by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan, and a Grant-in-Aid for scientific research (No. 13556033) from Ministry of Education, Culture, Sports, Science and Technology of Japan.

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(Received July 2004, Accepted December 2004)