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The Relative Distribution of Otoliths as a Means of Larval Fish Identification

Li Shu Chen[1,*] and Hong Young Yan[2]

[1]*National Museum of Marine Science and Technology – Preparatory Office, Keelung, Taiwan 202, R.O.C.*

[2]*School of Biological Sciences, University of Kentucky, Lexington, KY 40506-0225, USA*

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Li Shu Chen and Hong Young Yan (2001) The relative distribution of otoliths as a means of larval fish indentification. *Zoological Studies* **41**(2): 144-152. A recently developed auditory brainstem response (ABR) protocol has indicated that acoustically evoked brainwaves in fishes are species-specific. These brainwaves reflect the spatial distribution of auditory nuclei and the 3 auditory end organs along the ascending auditory neuronal pathways. Species-specific brainwaves suggest the hypothesis that the distribution of otoliths, which are closely coupled with sensory hair cells whose evoked response forms the 1st wave of the ABR, should also be species-specific. Eight species of marine fish larvae (belonging to 8 genera and 4 families) collected from Taiwan and Texas, USA were cleared in trypsin and examined microscopically with double polarization filters. Three indices (inter-utricular ratio, IUR; inter-saccular ratio, ISR; and inter-lagena ratio, ILR) were developed to measure the ratio of inter-otolithic to inter-orbital distances. IUR and ISR succeeded in separating species, but ILR was not as useful because of late development of the lagena. This study supports the hypothesis that the distribution of otoliths in fish larvae differs among species.
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The vestibular and auditory sensory modalities of fish are encoded by 3 major otolithic endorgans: the utricle, the saccule, and the lagena. Within each otic chamber resides an otolith: the lapillus (utricle chamber), the sagitta (saccule chamber), and the astericus (lagena chamber). Each otolith is lined with patches of sensory epithelia composed of sensory hair cells and supporting cells. An otolithic membrane provides a mechanical linkage between the otolith and the ciliary bundles of sensory hair cells where acoustic and vestibular stimuli are encoded. The otoliths of adult fish are calcareous structures of characteristic shapes and sizes and are species-specific (Helfman et al. 1997). One of the otoliths in particular, the sagitta, has been used as a major character in taxonomic studies of adult salmon (Casteel 1974), sciaenids (Chao 1978), neopterygian marine fishes (Hecht 1978), and fossil fishes (Huygeraert and Nolf 1979). Since the discovery of daily growth rings in otoliths of larval fish (Pannela 1971 1980), great interest has been focused on age, growth, mortality, life histories, migration, recruitment, and settlement of larvae from the information retrieved from the larval otoliths of American and European eels (*Anguilla rostata*, *A. anguilla*) (Wang and Tzeng 2000), anchovies (*Encrasicholina punctifer*, *Engraulis japonicus*) (Wang and Tzeng 1999), Atlantic herring (*Clupea harengus*) (Townsend and Graham 1981, Lough et al. 1982), Atlantic mackerel (*Scomber scombrus*) (Kendall and Gordon 1981), burbot (*Lota lota*) (Fischer 1999), chinook salmon (*Oncorhynchus tshawytscha*) (Zhang and Beamish 2000), grey mullet (*Mugil cephalus*) (Chang et al. 2000), haddock (*Melanogrammus aeglefinus*) (Gallego et al. 1999), Japanese eel (*Anguilla japonica*) (Tzeng and Tsai 1994, Tzeng et al. 1994, Tzeng 1995a, b, Cheng and Tzeng 1996), nehu (*Stolephorus purpureus*) (Struhsaker and Uchiyama 1976), northern anchovy (*Engraulis mordax*) (Methot and Kramer 1979), Pa-

*To whom correspondence and reprint requests should be addressed. Tel: 886-2-24631841 ext. 301. Fax: 886-2-24627716. E-mail: Lschen@mail.ntou.edu.tw

cific hake (*Merluccius productus*) (Bailey 1982), pleuronectid (*Parophrys vetulus*) (Laroche et al. 1982), red drum (*Sciaenops ocellatus*) (Rooker et al. 1999), rockfish (*Sebastes thompsoni*) (Kokita and Omori 1999), weak fish (*Cynoscion regalis*) (Thorrold et al. 1998, Paperno et al. 2000) and other marine (Brothers et al. 1976 1983, Lou and Moltschaniwdkyj 1992, Wilson and McCormick 1997 1999) as well as freshwater fishes (Claramunt and Wahl 2000).

However, to date, otoliths have rarely been used for larval species identification. Constraints of the limited use of otolith for species identification are: 1) small size, 2) simpler form, and 3) apparent lack of obvious distinguishing external features (Brothers 1984). Some progress has been made by examining crystallographic, mineralogical, and chemical levels (Lowenstan and Fitch 1978 1981, Lowenstan 1980 1981), amino acid composition (Degens et al. 1969), and trace elements analysis (Papadopoulou et al. 1978 1980, Gauldie et al. 1980) of adult otoliths as potential characters for taxonomic use. The direct application of these techniques to larval fish, however, is largely hampered by the small size of larval otoliths which renders analyses difficult or next to impossible.

Recently an auditory brainstem response (ABR) recording technique was developed to investigate acoustically evoked brainwaves from ascending auditory neuronal pathways of fishes (Kenyon et al. 1998, Ladich and Yan 1998, Yan 1998, Yan and Curtsinger 2000, Yan et al. 2000, Scholik and Yan 2001). The ABR is a non-invasive far-field recording technique which acquires brainwaves generated from a series of neurogenerators upon the reception of acoustic stimuli from 1st-order receptors (e.g., sensory hair cells of the inner ear) along ascending auditory pathways to higher brain centers (e.g., the auditory cortex). The acoustically evoked brainwaves from bluegill sunfish (*Lepomis macrochirus*) (Rigedon and Yan 1997), goldfish (*Carassius auratus*), the oscar (*Astronotus ocellatus*) (Kenyon et al. 1998), croaking gourami (*Trichopsis vittata*), pygmy gourami (*Trichopsis pumila*), dwarf gourami (*Colisa lalia*), paradise fish (*Marcopodus opercularis*), blue gourami (*Trichogaster trichopterus*) (Ladich and Yan 1998), kissing gourami (*Helostoma temminckii*) (Yan 1998), toadfish (*Opsanus tau*) (Yan et al. 2000), mormyrid weakly electric fish (*Brienomyrus brachyistius*) (Yan and Curtsinger 2000), and fathead minnow (*Pimephales promelas*) (Scholik and Yan 2001) have been demonstrated to be species-specific (Yan et al. 1997). Each acoustically evoked brainwave indicates the summation of neuronal activities of neurons along ascending auditory pathways, and the species specificity of the brainwave reflects the unique architecture of the neuronal network involved in the generation of acoustically evoked brainwaves. The 1st peak of the waveform is generated from the 3 hearing endorgans on each side of the inner ear and is also species-specific among many mammalian species examined (Hall 1992). It is known that taxonomical characters are variations of a homologous structure, and to be useful, they must show some variations in the taxon under study (Helfman et al. 1997). The species specificity of even the 1st peak of the acoustically evoked brainwave points to the possibility that the relative distribution of the otolithic organs of the fish may be species-specific and hence can be used as a valid character for use in systematic identification.

Otoliths of fish larvae are small and are relatively transparent under examination with a conventional dissecting microscope, which renders localization of these tiny otoliths difficult. Because of their calcareous nature and amino acid composition, they possess birefringence properties. With the aid of rotating polarization filters, transparent otoliths can be made to shine brightly against a darkened background when the phase retardation is set to $1/2\lambda$ (λ: wavelength of light used) (Slayter 1970, Rawlins 1992). Such a simple arrangement of polarization microscopy makes it easy to localize and to map the relative distribution of otoliths.

The purpose of this study was to test the hypothesis that the relative distribution of otoliths of larval fish can be used as a valid character for identification use. The application of double-polarization microscopy also helps to accelerate the mapping and measuring of the distribution of otoliths, hence shortening the time needed for species identification. Eight species of larval fish of a very divergent group of 4 families and 8 genera were compared to test the hypothesis.

MATERIALS AND METHODS

Fish larvae were collected from waters of Taiwan and Texas (see Table 1 for species, family, sampling sites, and sample size). Upon collection, the specimens were preserved in 70% ethyl alcohol. The trypsin enzyme method (Taylor 1967) was used to clear the specimens to facilitate the visualization of otoliths. Cleared specimens were examined by double-polarized microscopy as described below.

Double-polarization filter setup

A conventional dissecting microscope (Olympus SZX12) with a tungsten light source from underneath the stage was fitted with two 48-mm (diameter) rotary type polarized filters. The 1st polarizer was mounted directly underneath the glass stage (Fig. 1). The 2nd polarizer was mounted under the objective lens of the scope. The larval specimen was mounted onto a transparent Sylgard 184 silicone elastomer plate (Dow Corning, Midland, MI) with 00-size insect pins and immersed in 100% glycerin, i.e., the storage solution of cleared specimens. With the tungsten light on, the 2nd polarizer was rotated to align the incoming light which has passed through the 1st polarier to yield a darkened background with brightened otoliths (Fig. 2a).

Comparison of the relative distribution of otoliths

The relative distances of 3 pairs of otoliths of each species were calculated by 3 indices: IUR = (UU/IOD) x 100% (IUR: inter-utricular ratio; UU: distance between the 2 utriclar otoliths, the lapillus; IOD: inter-orbital distance), ISR = (SS/IOD) x 100% (ISR: inter-saccular ratio; SS: distance between the 2 saccular otoliths, the sagitta), ILR = (LL/IOD) x 100% (ILR: inter-lagena ratio; LL: distance between the 2 lagena otoliths, the astericus) (see Fig. 2b for measurements). The IOD was used as a common denominator to standardize all indices measured, a common practice used for morphometric measurements in ichthyological studies (Helfman et al. 1997). All distance measurements were made with the aid of an ocular micrometer and were calibrated with a stage micrometer. Boxplot diagrams (Hubbs and Hubbs 1953) were used to illustrate the range, mean, and standard error of each index. The Wilcox two-sample test was used to compare the mean values of indices obtained between red drum, kingfish, and banded drum. Due to limited sample sizes, no statistical test was applied to the remaining 5 fish species.

RESULTS

A total of 38 individuals representing 4 families, 8 genera, and 8 species was used in the study (Table 1). Most of the species have lapillus,

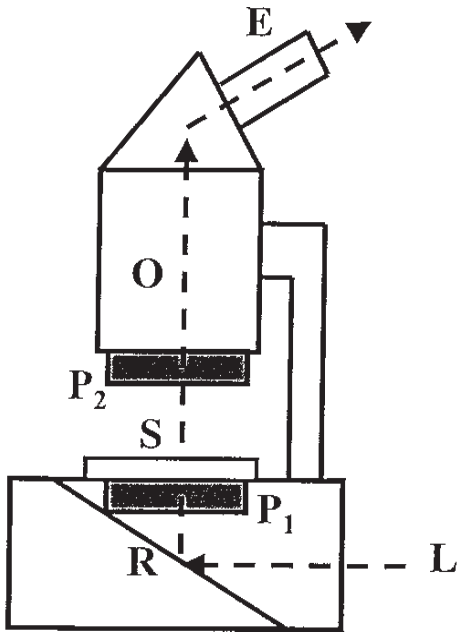

Fig. 1. Schematic diagram showing the optical path of double-polarized dissecting microscopy used to view larval otoliths. L: light source; R: reflecting mirror; P_1: 1st rotary polarizer; S: sample on top of a stage; P_2: 2nd rotary polarizer; O: objective lens: E: eyepiece. Arrows indicate the pathway of the light.

Table 1. Families, species, sampling sites, and numbers of individuals (sample size) of larvae used in the study

Family	Species	Sampling site	Sample size
Blennidae	*Istiblennius dussumieri*	Patozi, Taiwan	3
Leiognathidae	*Gazza minuta*	Tanshui, Taiwan	2
	Leiognathus nuchalis	Tanshui, Taiwan	2
Pomacentridae	*Abudefduf vaigiensis*	Patozi, Taiwan	3
Sciaenidae	*Cnyoscion nebulosus*	Port Aransas, TX	2
	Larimus fasciatus	Port Aransas, TX	6
	Menticirrhus americanus	Port Aransas, TX	5
	Sciaenops ocellatus	Port Aransas, TX	15

sciaenid species has its own unique mean value of ISR (Fig. 4, lower panel). In terms of the range of ISR values, that of red drum does not overlap with that of banded drum, while it does overlap with the values of spotted seatrout and kingfish. Wilcox two-sample test results show significant differences in mean values between red drum and kingfish (t_S = 6.34, $P < 0.01$), red drum and banded drum (t_S = 9.86, $P < 0.01$), and kingfish and banded drum (t_S = 4.32, $P < 0.01$). The ISR values of *Gazza minuta* do not overlap with those of *Istiblennius dussumieri,* but overlap slightly with those of *Leiognathus nuchalis*. There is no overlap of ISR between *Istiblennius dussumieri* and *Leiognathus nuchalis* (Fig. 4, upper panel).

The IUR and ISR measurements of 4 sciaenids (red drum, spotted seatrout, kingfish, and banded drum) (lower panels of Figs. 3 and 4) and Wilcox two-sample test results clearly indicate that these 2 measurements are species-specific. Within the family Leiognathidae, the ISR value of *L. nuchalis* differs from that of *G. minuta*; while IUR values of these 2 species may overlap, the mean value of *G. minuta* is larger than that of *Leiognathus nuchalis*, another indication of species specificity of the spatial arrangement of otolithic organs. The IUR and ISR values of blenny (*I. dussumieri*) overlap with those of damselfish (*A. vaigiensis*), and using only these ratios as the sole character for identification may pose some difficulties. However, distinct differences in the external morphology between the 2 species easily minimize possible confusion.

DISCUSSION

Traditionally the identification of fish is based entirely or mainly on the morphological structures of adults (Hubbs and Lagler 1964). It was not until recently that early life history (ELH) stages have been used as main characters for systematics use (see review by Cohen 1984 and extensive work cited in Moser et al. 1984). The taxonomic characters used during embryonic stages include diameter of developing embryos, number and size of oil globules, distribution of oil globules, width of the perivitelline space, shape and size of the yolk, and degree of yolk segmentation (Moser and Ahlstrom 1970, Moser 1972, McGowan and Berry 1984). The main characters used for larval fish identification include: 1) meristic counts such as the number of fin rays, vertebrae, and myomeres (Gordon et al. 1984), and 2) morphometric measurements of larvae including the ratios of head length to body depth, head width to head length, eye length to head length, and body depth to body length (Leis and Rennis 1983, Ahlstrom et al. 1984). Despite the usefulness of ELH in identifying larval species, the task itself is rather tedious and time consuming. Therefore, to identify a single key character that is species-specific would be of great value for quickly identifying targeted larval species for recruitment studies. The IUR and ISR indices developed in the present study provide an additional useful character for use in quick species identification. For instance, when both IUR and ISR ratios are taken into account, the 4 sciaenids can easily be separated by these values. This is even more valuable considering that all 4 sciaenids are found sympatrically and do not differ that much in their external morphology as well as body length. Cases like blenny and damselfish may overlap in their ratios; however, additional morphological characters such as a distinctive body shape and pigmentation patterns can aid in sorting out differences between species. A quick glance at the indices themselves also reveals the uniqueness of anatomical features of each species. For instance, both the IUR and ISR ratios of the red drum exceed 100% which clearly indicates that the cephalic region of the red drum is likely to have an anteriorly pointed triangular shape with inter-orbital distance narrower than the distance between inter-otolithic organs.

The advantage of using the indices developed herein is demonstrated in this study for quickly and accurately identifying larval fish. However, it is also imperative to point out that due to the nature of late formation of lagena otoliths (astericus), the ILR may not be as powerful as the values of IUR and ISR for identification use. The trypsin enzyme-clearing method was used in the present study to aid in easy visualization of otoliths, and it generally adds 1 additional day of effort for specimen preparation. However, for small-sized larvae (e.g., sciaenids: 3.1-9.0 mm), the location of otoliths can be identified easily even with just the use of double polarization filters without going through the enzyme-clearing process. The visualization of otoliths of larger size specimens, e.g., blennids (due to a thicker skull and muscle layers) especially can be greatly enhanced by clearing the samples.

In this study, only 2-dimensional distance values are measured and compared. The IUR, ISR, and ILR ratios used in the present study represent a simplified version of a Box-Truss measurement, which is a network of distance measures among homologous landmarks on a form, patterned as a series of contiguous quadrilaterals containing both internal diagonals (Brookstein et al. 1985). The full featured

Box-Truss method can be used to take into account distances among different otoliths and additional landmarks (e.g., eyes, tip of snout). However, in order to make the best use of the spatial distribution of otoliths as a powerful yet simple character for identification, a valid method of registering coordinates of 3-dimensional values of each otolith needs to be developed. This novel method also needs to incorporate a robust statistical method that can aid in discriminating the spatial distribution pattern of otoliths of each species. Future efforts should be directed to the development and refinement of registration of 3-dimensional spatial distribution of otoliths, so as to provide an even more powerful method of identifying larval fish. The limitation of sample size in 5 out of 8 species examined prevents further use of the *t*-test in comparing mean values between species. Nevertheless, significant statistical differences observed among 3 sciaenids clearly validate the merits of the methodology developed in the present study.

In conclusion, the data from this study support the hypothesis that the spatial distribution of otoliths is species-specific, and thus it can be used as a valid and practical character in larval fish identification. Furthermore, the use of readily available camera-type polarization filters is economically feasible and can provide a rather quick and convenient way of localizing otoliths in larval fish. The identification of larvae can become an easier task when used in conjunction with the indices developed in the study.

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