Correlation between Nuptial Colors and Visual Sensitivities Tuned by Opsins Leads to Species Richness in Sympatric Lake Victoria Cichlid Fishes

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Abstract

Reproductive isolation that prevents interspecific hybridization between closely related coexisting species maintains sympatric species diversity. One of the reproductive isolations is mate choice based on color signals (breeding color perceived by color vision). This is well known in several animal taxa, yet little is known about its genetic and molecular mechanism. Lake Victoria cichlid fishes are thought to be an example of sympatric species diversity. In the species inhabiting different light environments in rocky shore, speciation by sensory drive through color signals has been proposed by analyses of the long wavelength-sensitive (LWS) opsin gene and the male nuptial coloration. However, the genetic and molecular mechanism of how diversity of sympatric species occurring in the same habitat is maintained remains unknown. To address this issue, we determined nucleotide sequences of eight opsins of six sympatric species collected from a sandy–muddy shore—an ideal model system for studying sympatric species. Among eight opsins, the LWS and RH1 alleles were diversified and one particular allele is dominant or fixed in each species, and we propose that this is due to natural selection. The functions of their LWS alleles were also diversified as shown by absorption measurements of reconstituted visual pigments. To analyze the relationship between nuptial coloration and the absorption of LWS pigments, we systematically evaluated and defined nuptial coloration. We showed that the coloration was species specific with respect to hue and significantly differentiated by the index values of hue (dominant wavelength: $\lambda_d$). The $\lambda_d$ value of the male nuptial coloration correlated with the absorption of LWS pigments from all the species, suggesting that reproductive isolation through mate choice using color signals may prevent sympatric interspecific hybridization, thereby maintaining the species diversity in sympatric species in Lake Victoria.

Key words: opsin, color vision, cichlid fish, nuptial color, reproductive isolation, sympatic species richness.

Introduction

As species first begin to diverge and indeed after divergence, the identity of each newly emerged species is maintained by reproductive isolation through elimination of gene flow (Coyne and Orr 2004). Reproductive isolation is categorized as pre- or postzygotic. One of the mechanisms producing pre-zygotic reproductive isolation between sympatric species is interspecific mate choice, by which animals select a conspecific mating partner (Coyne and Orr 2004). Interspecific mate choice through color signals (breeding color perceived by color vision) is well studied; it has been suggested that it is important in maintaining sympatric species, and has been reported among sympatric closely related species in several animal taxa. For example, the diversification of coloration and color vision of lizards, birds, butterflies, and fishes is particularly remarkable (Boughman 2001; Jiggins et al. 2001; Roulin 2004; Seehausen et al. 2008; Losos 2009). However, little is known about the molecular and genetic mechanisms of mate choice through color signals for the establishment of reproductive isolation in such sympatric species.

The primary determinant of visual sensitivity in color vision is the absorption of photons by the visual pigments in retinal photoreceptor cells. Vertebrate visual pigments consist of a light-absorbing component (the chromophore) and a protein moiety (the opsin) (Dratz et al. 1985; Shichida and Imai 1998). Absorption spectra of visual pigments depend on the interactions between the chromophore and the opsin, and a change in the absorption spectrum depends on the properties of the chromophore (Harosi 1994; Parry and Bowmaker 2000) and amino acids interacting with the chromophore (Yokoyama 2000; Takahashi and Ebrey 2003; Hunt et al. 2003).
et al. 1997, 2008; Seehausen and van Alphen 1998) but also for mate choice (Fryer and Iles 1972; Seehausen et al. 2000). A2 retinal shifts the absorption of visual pigments toward longer wavelengths compared with A1 retinal (Whitmore and Bowmaker 1989). Both A1 and A2 visual pigments are localized in a photoreceptor cell, and the light absorbance of a photoreceptor cell depends on the ratio of A1 and A2 visual pigments. Moreover, the diversity of color vision by amino acid replacements in opsins has been well documented in vertebrates (Yokoyama 2000).

One of the most well-known examples of reproductive isolation by mate choice using coloration and color vision is cichlid species in Lake Victoria. It had been said that Lake Victoria has more than 500 endemic cichlid species (Seehausen 1996). They are thought to have experienced explosive adaptive radiation during a very short evolutionary period because Lake Victoria dried up at the end of the Pleistocene and was refilled only 15,000 years ago (Johnson et al. 2000). Although they exhibit a high diversity of species, genetic differentiation among species is extremely low (Nagl et al. 1998, 2000; Terai et al. 2004). Differentiation in one opsin, long wavelength-sensitive (LWS) gene, could represent the genetic differences (Terai et al. 2002, 2006; Carleton et al. 2005; Seehausen et al. 2008) that underlie species differentiation in this group (Terai et al. 2006; Seehausen et al. 2008).

African cichlids have eight opsins genes: one is for scotopic vision (RH1) (Sugawara et al. 2002) and the others are for color vision (SWS1, SWS2A, SWS2B, RH2Aα, RH2Aβ, RH2B, and LWS) (Parry et al. 2005; Spady et al. 2006; Hofmann et al. 2009). The sensitivities of opsins are as follows: scotopic vision (RH1), ultraviolet–violet-light sensitive (SWS1), blue-light sensitive (SWS2A and SWS2B), medium-light sensitive (RH2Aα and RH2Aβ), and medium- to red-light sensitive (LWS). Lake Victoria cichlids mainly express SWS2A (and SWS2B) for short-wavelength light, RH2A (putative RH2Aβ and/or RH2Aβ) for middle wavelengths, and LWS for long wavelengths (Hofmann et al. 2009). Cichlid vision is important not only for mate choice (Fryer and Iles 1972; Seehausen et al. 1997, 2008; Seehausen and van Alphen 1998) but also for food acquisition (Hori et al. 1993; Kohda and Horii 1993; Kawanabe et al. 1997).

In Lake Victoria, many species coexist in the same areas. Lake Victoria cichlids could potentially interbreed without infertility (Seehausen et al. 1997) under the condition when interspecific mate choice is disallowed (Crapon de Caprona and Fritzsch 1983; Crapon de Caprona 1986) probably due to lack of postzygotic reproductive isolation. In nature, instead of postzygotic reproductive isolation due to infertility of crosses, pre-zygotic reproductive isolation through mate choice by color signals is thought to eliminate gene flow among Lake Victoria cichlid species. In rocky shore habitat, the reproductive isolation through female mating preference to the nuptial coloration of conspecific male due to their color vision plays an important role in preventing interspecific hybridization (Seehausen et al. 1997, 2008; Seehausen and van Alphen 1998; Maan et al. 2006). Through analyses of LWS and nuptial coloration in Lake Victoria cichlids, we recently showed that adaptation of the sensory system (LWS opsin) to different environments drives the divergence of mating signals and leads to reproductive isolation (Terai et al. 2006; Seehausen et al. 2008). On the other hand, more than one-half of the shoreline in the southern part of Lake Victoria is sandy–muddy and inhabited by many cichlid species sympatrically (Witte et al. 1992). At such sandy–muddy shores, there are fewer structures (i.e., rocks and woods) than at rocky shores, and the species live in the same ambient light environment with no physical barriers. These species are an ideal model system for understanding the mechanism of the reproductive isolation among coexisting species. However, little is known about the visual sensitivity (opsins) and the nuptial coloration diversity of these species. In this study, we used six coexisting species from Mwaburugu in Lake Victoria, an ideal area to study sympatry. We analyzed their color vision (opsins) and nuptial colorations and demonstrate that the opsin tuning correlates with the nuptial coloration characteristic.

Materials and Methods

Sample Information

The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology. We used six Lake Victoria cichlid species: *Haplochromis* sp. “macula,” *Haplochromis pyrrhocephalus* Witte and Witte-Maas (1987), *Platyaeniodus degeni* Boulenger (1906), *Haplochromis* sp. “thick skin,” *Haplochromis riponianus* (Boulenger 1911), and *Haplochromis fischeri* Sideer (2008). *Haplochromis fischeri* was misidentified as *Haplochromis sauvagei* (Pfeffer 1896) in Greenwood (1957), Barel et al. (1977), and Seehausen (1996) (see Seegers 2008). These species are widely distributed in Lake Victoria (Greenwood 1956; Witte and van Oijen 1990; Seehausen 1996; Mizighani et al. 2010) and inhabit Mwaburugu at the east end of Speke Gulf (Fig. 1A). Previous work and our field research suggested the depth distributions of these species inhabiting several localities: *H. pyrrhocephalus* inhabit surface to middle layer (van der Meer and Bowmaker 1995); *H. riponianus* is demersal species (Seehausen 1996); *H. sp. “thick skin,” P. degeni, H. sauvagei,* and *H. sp. “macula” are demersal species supported by our field research and previous works (Witte 1984; Witte et al. 1992). In Mwaburugu, however, the depth distributions of these species were very close (caught by a single net), and we could not identify their depth distributions. All specimens were collected from Mwaburugu by netting (1.5-m height) at 1- to 3-m depth. All fish were collected by M.A. and Dr. Shinji Mizoiri (M.A.) in 2004–2006. Identification of all specimens was verified by M.A. and S.M.

DNA Sequencing and Assembly

We extracted genomic DNA from caudal fin, pectoral fin, or muscular tissues using DNeasy Blood & Tissue kit (Qiagen). All tissues were dissected and kept in 100% ethanol until use. Cichlid opsin genes (SWS1, SWS2A, SWS2B, RH2Aα, RH2Aβ, RH2B, LWS, and RH1) and the control region of mitochondrial DNA (mtDNA) were sequenced as described (Sugawara et al. 2002; Carleton et al. 2005; Terai et al. 2006; Maeda et al. 2009).

3282
with minor modifications. Briefly, the DNA fragments including opsin genes or mtDNA were amplified by PCR primers specific to each gene (see supplementary file S1, Supplementary Material online) in the PTC-100 Programmable Thermal Controller (MJ Research). SWS2A, SWS2B, RH2B, and LWS were amplified by two rounds of PCR. The first round amplified nearly full length of these genes using a pair of first PCR primers specific to each gene (see supplementary file S1, Supplementary Material online). The second round of PCR used the first PCR products as template, and amplified a set of two overlapping fragments using a pair of second PCR primers specific to each gene (see supplementary file S1, Supplementary Material online). The PCR program for opsin gene and mtDNA amplification consisted of a denaturation step for 3 min at 94°C, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1.5–5 min (extension time specific for each gene) at 72°C. The PCR products were purified and their sequences were determined by direct sequencing using the Applied Biosystems Automated 3130 Sequencer. When the protein-coding sequences of LWS, RH2Aβ, and RH1 gene included heterogeneous sites, we subcloned the PCR products into the pGEM-T plasmid vector (Promega) and determined the sequences of several clones to obtain the haplotype information. The primer sequences are described in supplementary file S1, Supplementary Material online. To determine the sequences of 25-kb DNA fragments including three opsin genes (SWS2A, SWS2B, and LWS) and its flanking regions from Mwaburugu cichlids, we separated the region into seven overlapping fragments: 1) flanking region upstream of SWS2A (2.3 kb), 2) SWS2A gene (2.1 kb), 3) flanking region between SWS2A and SWS2B (4.1 kb), 4) SWS2B gene (2.4 kb), 5) flanking region downstream of SWS2B (0.9 kb), 6) 10.5-kb region including LWS (Terai et al. 2006), and 7) downstream of 5) (3.1 kb). Determination of 2) SWS2A, 4) SWS2B, and 6) LWS was performed as noted...
earlier. The sequences flanking 6) were determined as described (Terai et al. 2006) with minor modifications. To determine the sequences of 1), 3), 5), and 7), we designed primers for PCR and sequencing based on the DNA sequence of the BAC clone including LWS as described (Terai et al. 2006). The BAC clone sequences of 1), 3), 5) were determined by direct sequencing using DNA from the BAC clone as a template. For 7), the BAC clone was digested with Xba I and subsequently subcloned into pUC18. The Xba I fragment including the downstream region was screened and directly sequenced. We amplified and determined the fragment including the downstream region was screened (Terai et al. 2006).

The DNA fragments of 3), 6), and 7) were amplified by long PCR in the first PCR step as described (Terai et al. 2006). The DNA fragments of 3), 6), and 7) were amplified by long PCR and directly sequenced. We amplified and determined the fragment including the downstream region was screened (Terai et al. 2006).

Determination of the sequences flanking RH1 (total 7 kb: 3-kb upstream, RH1, and 3-kb downstream) followed previous work (Nagai et al. 2011). We designed primers to amplify and determine the DNA sequence based on the BAC clone sequence including RH1 (Nagai et al. 2011). The 7-kb DNA fragments containing RH1 were amplified by long PCR and determined from 44 individuals of 4 species: H. pylrocephalus (N = 10), H. pyrrocephalus (N = 10), H. fischeri (N = 13), and P. degeni (N = 10). We could not amplify the DNA fragments downstream of LWS from one specimen (I.D.: 00325) of P. degeni and thus removed downstream of LWS from further analysis. The DNA fragments of 3), 6), and 7) were amplified by long PCR in the first PCR step as described (Terai et al. 2006).

Determination of the sequences flanking RH1 (total 7 kb: 3-kb upstream, RH1, and 3-kb downstream) followed previous work (Nagai et al. 2011). We designed primers to amplify and determine the DNA sequence based on the BAC clone sequence including RH1 (Nagai et al. 2011). The 7-kb DNA fragments containing RH1 were amplified by long PCR and determined from 44 individuals of 4 species: H. sp. “macula” (N = 10), H. pyrrocephalus (N = 10), H. fischeri (N = 13), and P. degeni (N = 11). The primer sequences and positions are described in supplementary file S1 and supplementary figure S1, Supplementary Material online. All sequences obtained were assembled with GENETYX version 10.0.1 or ATGC version 6.0.2 (Genetyx Corporation).

Population Genetic Analysis
We calculated FST and r2 from eight opsin genes (using protein coding sequences) and mtDNA loci by DnaSP version 5.1.0 (Librado and Rozas 2009). For mtDNA, population subdivision was estimated based on FST, and significance of population subdivision was evaluated by the Snn-test statistic (Hudson 2000). We performed sliding window analysis to calculate approximate values for FST (Da/Dxy [Slatkin 1991]), the average number of nucleotide differences (K) and π in 1,500-bp windows, sliding the window in steps of 10 bp throughout the 25-kb LWS region, and in 500-bp windows, sliding the window in steps of 10 bp throughout the 7-kb RH1 region.

To detect selection pressure acting on LWS and RH1 of four Mwaburuguruguru species, we performed three neutrality tests: McDonald test (McDonald 1998), Tajima’s D test (Tajima 1989), and the MLHKA test (Wright and Charlesworth 2004). We performed the McDonald test for heterogeneity across a region of the DNA sequences in the ratio of polymorphism to divergence as described (Terai et al. 2006; Seehausen et al. 2008). Briefly, we performed this test using DNA Slider program for Mac OS X provided by J.H. McDonald (http://udel.edu/~mcdonald/aboutdnaslider.html; last accessed June 26, 2012), with the recombination parameters set to 2, 4, 10, and 32, and the number of replications set to 1,000 replicates. In McDonald test, we used the sequences for sliding window analysis in both LWS (25 kb) (H. sp. “macula”: N = 10; H. pyrrocephalus: N = 10; H. fischeri: N = 13; P. degeni: N = 10) and RH1 (7 kb) (H. sp. “macula”: N = 10; H. pyrrocephalus: N = 10; H. fischeri: N = 13; P. degeni: N = 11). The McDonald test calculated P-values for each test condition (each recombination parameter) and we used the largest P-value for significance tests.

Tajima’s D values were calculated by DnaSP at 11 loci as described (Breen et al. 2009): SWS1 (1.3 kb), SWS2A (1.9 kb), SWS2B (2.3 kb), RH2Aa (1.5 kb), RH2Ab (1.4 kb), RH2B (2.9 kb), LWS (2.2 kb), RH1 (1.1 kb for H. sp. “macula,” P. degeni, and H. fischeri, and 4 kb including upstream and downstream flanking sequences for H. pyrrocephalus), LWS downstream region (3.1 kb), RH1 upstream region (1.9 kb), and RH1 downstream region (1.3 kb). We used the following number of individuals for this test: H. sp. “macula” (N = 23), H. pyrrocephalus (N = 20), H. fischeri (N = 13), and P. degeni (N = 10 for downstream of LWS; N = 11 for the other loci). We obtained the associated one-tailed P-values for Tajima’s D by computing 10,000 coalescent simulations based on segregating sites from the observed data and assuming free recombination in DnaSP, as described previously (Breen et al. 2009).

The MLHKA test followed previous work (Wright and Charlesworth 2004) and was performed using the MLHKA program provided by S.I. Wright (http://labs.eeb.utoronto.ca/wright/Stephen_I_Wright/Programs.html; last accessed June 26, 2012). The MLHKA test assesses departure from neutrality at a test locus compared with reference sequences (neutral standards). The degree of increase or decrease of polymorphism caused by selection is measured as k index: k = 1, k < 1, and k > 1 indicate neutral evolution, evolution under selection toward decreasing nucleotide diversity (i.e., selective sweep), and evolution under selection increasing nucleotide diversity (i.e., balancing selection), respectively (Wright and Charlesworth 2004). For the MLHKA test, we used 4 loci (SWS1, RH2Aα, RH2Aβ, and RH2B) as reference sequences and test 7 loci (SWS2A, SWS2B, LWS, downstream of LWS, RH1, upstream and downstream of RH1). We used the following number of individuals for this test: H. sp. “macula” (N = 23), H. pyrrocephalus (N = 20), H. fischeri (N = 13), and P. degeni (N = 10 for downstream of LWS; N = 11 for the other loci). The number of segregating sites (S) Watterson’s estimator (θW) (Watterson 1975) and pairwise divergence between test and outgroup species (D) for 11 loci were calculated using DnaSP. To conduct the tests, we used 4 reference loci and 1 test loci and ran 100,000 simulations in the MLHKA program to compare the fit of a neutral model to a model specifying test loci in each species.

Based on the neighbor joining phylogenetic tree of the (7) region (except LWS) and the 7-kb region (except RH1) (see DNA Sequencing and Assembly section), we defined outgroup species for the McDonald and MLHKA test. For the LWS analysis, P. degeni was used for H. sp. “macula,” H. pyrrocephalus, and H. fischeri, and H. sp. “macula” was used for P. degeni. For the RH1 analysis, Trophus duboisi (Lake Tanganyika cichlid) was used for all four species. RH2Aα,
RH2B, and the 7-kb RH1 region of T. duboisi were sequenced as mentioned earlier. The other opsin sequences of T. duboisi (Spady et al. 2005) are as follows: The accession number of SWS1, SWS2A, SWS2B, and RH2Aβ are AY775099, AY775073, AY775082, and AY775089, respectively. Because we performed multiple tests for neutrality using combination of multiple species (4 species) and multiple loci (McDonald test: 2 loci; MLHKA test: 7 loci; Tajima’s D test: 11 loci), we adjusted level of significance by sequential Bonferroni correction (Rice 1989) for each test (significant level for McDonald test: P < 0.006 [0.05/8]; for MLHKA test: P < 0.001 [0.05/28]; for Tajima’s D test: P < 0.001 [0.05/44]).

Reconstruction and Measurement of Absorption Spectra of Visual Pigments
Production, reconstruction, purification, and measurement of the visual pigments were performed as described (Ueyama et al. 2002; Terai et al. 2006) with minor modifications. Briefly, the sequences of LWS (M3 and Py alleles), RH1 (104V and 104I alleles), SWS2A, SWS2B, RH2Aα, and RH2Aβ (β1–β5 alleles) were amplified by RT-PCR using total RNA extracted from eyes of Lake Victoria cichlids as a template with a pair of PCR primers (supplementary file S1, Supplementary Material online) designed to produce a fusion protein with a FLAG-tag (Sigma-Aldrich) at its C terminus. The LWS-Sp construct was made from the LWS-P construct (Seehausen et al. 2008) by PCR-based mutagenesis. The amplified DNA fragments were digested with restriction enzymes and cloned into the expression vector pMTS (Khorama et al. 1988) or pFLAG-CMV-5a (Sigma-Aldrich). The visual pigments were reconstituted with A1 retinal and A2 retinal. A2 retinal was synthesized as described (Wada et al. 2008). Absorption spectra of the pigment solutions (in the presence of hydroxylamine, <100 mM) before and after photobleaching were recorded using a spectrophotometer (UV-2400, Shimadzu, Japan). The measurements were taken 5–30 times before and after photobleaching. We determined the mean peak spectral values (maximum absorption spectra: λmax) and standard errors from multiple preparations and measurements for each pigment. Although we measured A2 pigments of SWS2B, we could not calculate the accurate λmax value the same way as the other opsin pigments because the absorption of SWS2B A2 pigments and free A2 retinal overlapped. All procedures after reconstitution of the pigments were performed under dim red or infrared light conditions with the vision of a digital video camera recorder (DCR-TRV8, Sony) in “night shot” mode or under complete darkness.

The GenBank accession numbers were as follows: SWS2A (AB666585), SWS2B (AB666652), RH2Aα (AB666719), RH2Aβ_β1 (AB667661), RH2Aβ_β2 (AB667773), RH2Aβ_β3 (AB667759), RH2Aβ_β4 (AB667719), RH2Aβ_β5 (AB667709), LWS_M3 (AB666974), LWS_Py (AB666943), LWS_Sp (AB666972), RH1_104V (AB667475), and RH1_104I (AB667476). We used these sequences for the constructs using opsin measurements without introns.

Digitization of Male Nuptial Color
As soon as possible after capture, fish were immobilized in a cuvette and photographs were taken. We took photographs of live males without any anesthesia. All photographs were taken by three digital cameras (FinePix S5200, FUJIFILM; DMC-FZ10, Panasonic; and Optio WP, Pentax) using auto exposure in daytime. Fish body color under the morning or sunset sunlight appeared distinctly reddish relative to those under day light because morning or sunset sunlight mainly included reddish (long wavelength) light caused by scattering bluish (short wavelength) light by the atmosphere. Therefore, we avoid taking photographs under such reddish light condition may cause misleading in further identification of fish color. To adjust color balance, the photographs were taken with high durability white tags (pieces of high durability white cloth in which I.D. numbers were described) as white standard. In this study, the photographs were saved as JPEG images with sRGB color mode and we analyzed colors of these images displayed on two PC monitors (both monitors were the same model, LL-T7A4-H, SHARP) with automatically color adjusted with sRGB color mode. On the photographs, some male fishes showed their bright colors and some lost them because of their rapid color changing. We only used the photographs with bright color for further analysis.

Our color analysis potentially included several points to be revised in the future. First, photographs were not taken under exactly the same condition such as different time in daytime and different camera models. Second, we lost data of some photographs, which include detailed information of condition of photographs (i.e., camera model). Although these points potentially lead to change of fish colors on the photographs from natural fish colors, we could adjust color balance using white standards. Third, photographs were saved as JPEG images. JPEG images loss color information due to algorithmic compression based on human color vision (Stevens et al. 2007), meaning that color information on the photographs was modified and limited rather than that of natural fish color. Our purpose of color analysis in the present study was to reveal whether there were tendency of linear relationship between λmax of LWS and average λd of lateral body coloration as described latter. Although it was tentative color analysis, our analysis could roughly capture the feature of correlation between LWS tuning and male colorations.

Based on the distribution pattern of male nuptial color according to previous work (Barel et al. 1977), we divided the fish body into 16 areas (1, cheek and snout; 2, operculum and dorsal head surface; 3, dorsum; 4, anterior margin of mid and ventral lateral body; 5, caudal peduncle and posterior margin of mid and ventral lateral body; 6, membrane of spiny dorsal fin; 7, membrane of dorsal fin soft ray; 8, maculate in posterior margin of dorsal fin membrane; 9, dorsal fin lappets; 10, ventral margin of caudal fin; 11, posterior end of caudal fin; 12, caudal fin membrane; 13, marginal of spiny pelvic fin; 14, marginal of pelvic fin soft ray; 15, marginal of spiny anal fin; and 16, marginal of anal fin soft ray). To evaluate male nuptial colors, we used the Munsell color (MC) chart (Munsell color system 2008, Japan Color
Enterprise Co., Ltd.) as a reference color standard. In the case of chromatic color, the MC was defined as three indices: Hue (Munsell Hue: MH), Value, and Chroma. In the case of achromatic color (white, black, or gray), the MC was defined by only Munsell Value (brightness of color), meaning that this MC lack MH index. We used the MC chart including 20 hues: SR, 10R, 5YR, 10YR, 5Y, 10Y, 5GY, 10GY, 5G, 10G, 5BG, 10BG, 5B, 10B, 5PB, 10PB, SP, 10P, SRP, and 10RP (R, G, B, and P indicate red, green, blue, and purple, respectively). In each area of the fish body, we compared male color of the photograph with the MC chart and selected the MC most similar to the fish color from the chart. The photographs were displayed on PC monitor as mentioned earlier, and the selections were performed under commercial wide-range fluorescent light in the laboratory. All MCs were assigned by M.A. and S.M. We used MCs from lateral body (fig. SA, area numbered 1–5, whose area numbers are equal to the areas named earlier) for further analysis because these five are distinct in coloration among species. Then, for each individual, we collected a total of 10 MCs from 5 body parts (fig. SA, 1–5 areas and 2 observers). We defined MC as the index of the fish color from 36 male individuals of the six Mwaburugu species: H. sp. “macula” (N = 11), H. pyrrhocephalus (N = 4), H. fischeri (N = 5), P. degeni (N = 7), H. sp. “thick skin” (N = 5), and H. ripionanus (N = 4). The specimens of H. pyrrhocephalus (4 of 4 individuals), P. degeni (2 of 7), and H. ripionanus (3 of 4) were collected in Mwaburugu. But we did not determine their LWS sequences because the specimens were fixed by 10% formalin. LWS sequences of the other specimens were determined as mentioned above. We classified all MCs into 20 groups according to their MH value, and calculated the rate that the number of each MH group accounted for the total number. If fish body color was achromatic color (white, black, or gray), MC of this body area was defined by only Munsell Value. Then, we removed this MC lacking MH from further color analysis, because our color analysis focused on only hue. For example, we performed color analysis using 70 body areas of P. degeni species (from 7 specimens, 5 body areas, and 2 observers). We defined 66 of 70 areas as chromatic color (MC including MH) and 4 areas as achromatic color (MC lacking MH). Then, we removed 4 MCs (lacking MH) and totally collected 66 MHs, and 31 of 66 MHs were counted in MH-SB group. Thus, MH-SB group account for 47% of the total. We performed Fisher’s exact test to evaluate the differentiation of the MH of lateral body among Mwaburugu species.

In addition, we translated the MC into x and y values of Commission Internationale de l’Eclairage (CIE) 1931 system (CIE-Yxy color space) using the transform table established previously (Wyszecki and Stiles 1982). The dominant wavelength (λd) was then calculated from the x and y values using CIE 1931 xy chromaticity diagram with standard illuminant C (Wyszecki and Stiles 1982) as described previously (Popov-Ralji et al. 2009). In addition to MC lacking the MH, in the case of obtaining a minus λd value, this MC was removed from further data analysis. The average as well as the median value of λd was estimated from the collection of the five λd values from five lateral body parts in all the individuals. To test differentiation of λd of lateral body color among six species, we also performed multivariate analysis of variance (MANOVA) using Wilks’ lambda result.

We calculated the Spearman’s rank correlation coefficient to evaluate the correlation between the λd and the λmax of LWS pigments. In Spearman’s rank correlation coefficient test, we estimated the ratio of A2 pigments from the λmax value of the microspectrophotometry (MSP) data (table 1) (Carleton et al. 2005). As mentioned in Introduction, a photoreceptor cell includes both A1 and A2 pigments. MSP can measure absorption spectra of single photoreceptor cell, meaning that absorption spectra measured by MSP is the sum of absorption spectra of A1 and A2 pigments with certain A1/A2 ratio. To estimate ratio of A1/A2 pigments in photoreceptor cells, firstly we constructed LWS standard absorption curve using LWS absorption curves (fig. 4). In each pigment (A1 and A2), we unified the peaks of the curves (λmax) of four LWS allele pigments (fig. 4, H, Sp, Py, and M3), and also unified the peak absorbance. To construct standard curves for each of A1 and A2 pigment, we averaged four absorption curves and smoothed them. The standard curves were adjusted λmax to 4 LWS alleles. Then, we used total eight absorption curves (four are for A1 and four are for A2) as template curves (the same curves with different λmax). For each LWS allele, we estimated λmax of the mixture of A1 and A2 pigments with certain A1/A2 ratio. For example, if A1/A2 ratio is 50/50, we construct the same peak absorbance curves for A1 and A2 pigments of one LWS allele, and construct one curve by A1 curve plus A2 curves. Then, the curve represent mixture of A1 and A2 curve with A1/A2 = 50/50 ratio. For each LWS allele, we estimated λmax of the mixture of A1 and A2 pigments with 11 A1/A2 ratio (A2 ratios: 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) and plotted A2 ratio versus λmax of the mixture of A1 and A2 pigments in each of 4 LWS allele. Using these plots, we estimated the A1/A2 ratio from MSP data. Absorption spectra of LWS alleles pigments measured by MSP in Lake Victoria cichlid species were reported (Carleton et al. 2005), and we estimated A1/A2 ratio of these MSP data. This estimation showed that Lake Victoria cichlids use the A2 pigments at a rate from 34% to 58%. Spearman’s rank coefficient test required rank value for λmax of LWS pigments estimated from each LWS allele with each A1/A2 ratio. We performed Spearman’s rank correlation test in several A1/A2 ratio (A2 ratios: 0%, 25%, 50%, 75%, and 100%).

In Fisher’s exact test and MANOVA test, we used the MCs and λd data defined by M.A. or S.M. Then, we performed the tests separately for each data set (M.A. or S.M.). All tests were performed by R 2.11.1 (R Development Core Team 2010).

Results and Discussion

LWS Allele-Dependent Diversity of Color Vision in Mwaburugu Cichlids

Rocky shores along Lake Victoria create various microenvironments such as the plane surface of rocks, the inside of rock crevices and the sidewall of vertical rocks. Ambient light environments vary, and the visual adaptations of Lake Victoria
Table 1. The \( \lambda_{max} \) Values of Visual Pigments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>A1 Pigment</th>
<th>A2 Pigment</th>
<th>D(^a)</th>
<th>( \lambda_{max} ) (nm) from MSP works</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \lambda_{max} ) (nm)(^b)</td>
<td>( \lambda_{max} ) (nm)(^b)</td>
<td>Pandamilia(^d)</td>
<td>Haplochromis(^e)</td>
</tr>
<tr>
<td>RH1</td>
<td>104V</td>
<td>502 ± 1</td>
<td>524 ± 1</td>
<td>22</td>
<td>503–506</td>
</tr>
<tr>
<td></td>
<td>104I</td>
<td>503 ± 1</td>
<td>523 ± 1</td>
<td>20</td>
<td>503–506</td>
</tr>
<tr>
<td>LWS(^f)</td>
<td>H</td>
<td>559 ± 1(^k)</td>
<td>604 ± 1(^k)</td>
<td>45</td>
<td>567–571</td>
</tr>
<tr>
<td></td>
<td>M3</td>
<td>553 ± 1</td>
<td>601 ± 1</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Py</td>
<td>551 ± 1</td>
<td>595 ± 1</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sp</td>
<td>549 ± 1</td>
<td>595 ± 1</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>SWS2A</td>
<td>—</td>
<td>457 ± 1</td>
<td>472 ± 1</td>
<td>15</td>
<td>451–456</td>
</tr>
<tr>
<td>SWS2B</td>
<td>—</td>
<td>430 ± 1</td>
<td>—</td>
<td>—</td>
<td>527–538(^b)</td>
</tr>
<tr>
<td>RH2A(^\beta)</td>
<td>( \beta_1 )</td>
<td>523 ± 1</td>
<td>546 ± 1</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \beta_2 )</td>
<td>523 ± 1</td>
<td>546 ± 1</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \beta_3 )</td>
<td>522 ± 1</td>
<td>547 ± 1</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \beta_4 )</td>
<td>524 ± 1</td>
<td>547 ± 1</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \beta_5 )</td>
<td>523 ± 1</td>
<td>546 ± 1</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)The \( \lambda_{max} \) values are indicated with their standard errors.

\(^b\)MSP data were measured by van der Meer and Bowmaker (1995) from four coexisting Haplochromis species in Lake Victoria cichlids: H. piceatus, H. argens, H. ishmaal, and H. pyrrhocephalus. Although opsin gene sequences and expression were not determined in this work, the MSP data putatively classified into four pigment types: RH1 (Rod), LWS, SWS (putative SWS2A and/or SWS2B), and MWS (putative RH2A and/or RH2A\(^\beta\)) groups.

\(^c\)Difference of \( \lambda_{max} \) between A1 and A2 retinal pigments.

\(^d\)MSP data were measured by Carleton et al. (2005) from five Pandamilia species in Lake Victoria cichlids: P. pundamilia, P. nyererei, P. azurea, P. "luanso," and P. "red head."

\(^e\)Three coexisting Haplochromis species: H. piceatus, H. argens, and H. ishmael.

\(^f\)\( \lambda_{max} \) of LWS pigments were shown in italics.

\(^g\)\( \lambda_{max} \) of putative RH2A pigments (probably included both RH2A and RH2A\(^\beta\) pigments).

rock cichlids to such variety of ambient environments with different transparencies and depths have been reported (Terai et al. 2006; Seehausen et al. 2008). On the other hand, sandy–muddy bottoms with no rocks, woods, or other structures are environmentally less diverse. Despite the relative uniform environment, a large number of cichlid species coexist in sandy–muddy shores (Witte et al. 1992). We considered that the diversification of the visual system in coexisting cichlid species from sandy–muddy shores might provide one explanation for the maintenance of species diversity in this environment. Thus, we chose to study cichlids living in Mwabarugu (fig. 1A), a consistently shallow area (2–3 m) with a sandy–muddy bottom and no physical barriers, located in the eastern region of Speke Gulf. Six species were collected from this area using nets (1.5-m height) at the same point and depth. According to this constant environment, we regarded these six species as sympatric species and investigated their visual diversification.

To ascertain whether the opsins of these sympatric species are diversified, we sequenced all eight opsin genes (RH1, SWS1, SWS2A, SWS2B, RH2A\(^\alpha\), RH2A\(^\beta\), RH2B, LWS) from 67 individuals of four coexisting species; H. sp. “macula” (N = 23), H. pyrrhocephalus (N = 20), P. degeni (N = 11), and H. fischeri (N = 13, see Materials and Methods section about species name). Then, we calculated indices of genetic diversity (\( \pi \)) and genetic differentiation (\( F_{ST} \)) using each opsin gene sequence from these species to reveal the diversity and differentiation of the genes between and within species. We also calculated \( \pi \) and \( F_{ST} \) for the D-loop region of mtDNA, which can be considered as a neutral marker. The \( F_{ST} \) values for LWS and RH1 between species showed obviously higher values (0.75–0.98) than those for the other opsin genes and mtDNA (<0.01–0.36) except for those for RH1 from the H. sp. “macula” and H. pyrrhocephalus (ma–py) species pair and the P. degeni and H. fischeri (de–fi) pair (fig. 2A). The \( \pi \) values for LWS and RH1 within each species were lower than those for RH2A\(_\alpha\) and RH2A\(^\beta\) (fig. 2B). In contrast, the \( \pi \) value for all the LWS sequences from four species (~0.0055; fig. 2B) was higher than that for LWS within each species. This value was also higher than those for other seven genes within each species as well as from four species (fig. 2B). These data suggest that the LWS alleles were species specific, whereas those of other opsins were shared among species.

We translated nucleotide to amino acid sequence and showed the presence of amino acid diversity, especially in LWS, RH1, and RH2A\(^\beta\), among the eight opsin genes (supplementary fig. S2, Supplementary Material online). To obtain more comprehensive data about this diversity, we added the sequences of LWS and RH1 from 27 individuals of 2 additional coexisting species (H. sp. “thick skin”: N = 23, and H. ripponius: N = 4) and further analyzed these opsins (supplementary fig. S2, Supplementary Material online). In LWS, 10 haplotypes with 21 polymorphic sites (7 synonymous and 14 nonsynonymous) were observed (supplementary fig. S3A, Supplementary Material online). In RH1, seven haplotypes with six polymorphic sites (two synonymous and four nonsynonymous) were observed (supplementary fig. S3B, Supplementary Material online). All the LWS haplotypes could be mainly
divided into four allele groups, named H, M3, SP, and Py, based on the amino acid sequence (fig. 1B) (HandM3 groups were previously described [Terai et al. 2006; Seehausen et al. 2008]). Similarly, we divided all the observed RH1 haplotypes into 2 allele groups (104V and 104I) based on a variable amino acid at position 104 (fig. 1C). Analyses on the frequencies of LWS and RH1 allele groups showed that each Mwaburugu species possesses a dominant allele for each LWS and RH1 ([LWS: 72–100%, RH1: 88–100%, fig. 1E and F]) except that of RH1 for H. sp. “thick skin” (haplotype number: 104V = 23, 104I = 23). In contrast to LWS and RH1, the allele groups of RH2A/C12 (1–11) were shared among the original four species (supplementary fig. S3, Supplementary Material online).

Next, we investigated the function of opsins. Although color vision is influenced by several signaling pathways (i.e., photon detection by photoreceptor pigments, transmittance of electron signals, and signal processing in brain) (Sharpe et al. 1999), the $\lambda_{\text{max}}$ shift of cone opsin pigments is thought to be one of the most influential factors to determine visual sensitivity in vertebrates (Yokoyama 2000). One of the problems in the reconstruction of cone opsin pigments is the unavailability and instability of A2 retinal. To overcome this problem, we performed a large-scale organic synthesis of A2 retinal and used this highly purified chromophore for the analysis (Wada et al. 2008). Then, we reconstituted LWS (from M3, Py, and Sp allele), RH1 (from 104V and 104I allele), and RH2A$\beta$ (from $\beta$1–5 allele), SWS2A, SWS2B, and RH2A$\alpha$ pigments in vitro with A1 retinal or A2 retinal and measured their absorption spectra.

The $\lambda_{\text{max}}$ values of LWS pigments of four alleles with A1 ranged from 549 to 559 nm, and those with A2 were red-shifted and ranged from 595 to 604 nm (fig. 3A and table 1). The shifts by chromophore usage (A1 or A2) differed among the pigments reconstituted from different LWS alleles, ranging from 44 to 48 nm (table 1). These are the first comprehensive data of the $\lambda_{\text{max}}$ values of LWS opsin using A2 pigments in fish except for our original experiment (Terai et al. 2006). In Lake Victoria cichlids, it was estimated that a 4-nm blue shift of the $\lambda_{\text{max}}$ for LWS pigments leads to a 10% reduction in sensitivity to red light (Carleton et al. 2005). We compared the $\lambda_{\text{max}}$ values of every combinations between the 4 LWS alleles with A1 (6 pairs: H vs. M3, H vs. Py, H vs. Sp, M3 vs. Sp, M3 vs. Py, and Py vs. Sp). In the case of A2, we did the same analysis (six pairs). Among these 12 pairs, 8 pairs showed differences in the $\lambda_{\text{max}}$ of LWS pigments of ≥4 nm (fig. 3A and table 1), indicating the diversification of light sensitivity in longer wavelength light.

Our experiments also demonstrated that SWS2A, SWS2B, and RH2A$\alpha$ pigments absorbed blue-light, violet- to blue-light and medium-light, respectively, as shown in figure 3D–F and table 1. Activities of these absorbing functions were confirmed by comparison with those of other cichlid fishes (Parry et al. 2005; Spady et al. 2006). In the case of the 2 RH1 alleles (104V and 104I), although one RH1 allele was dominant in each species, there was no difference in their
absorption spectra (fig. 3B and table 1). In the case of the five RH2Aβ alleles (β1–5), whose sequences are shared among species, there was also no difference in their absorption spectra (fig. 3C and table 1). In contrast, the diversity and differentiation of LWS in sequence and the differences of LWS pigments in λ_max (light absorbing function) are remarkable, providing the possibility that sympatric species have LWS allele-dependent diversity of color vision.

LWS Diversity among Species Is Driven by Natural Selection

What has driven the diversity of LWS? Terai et al. (2006) and Seehausen et al. (2008) reported that natural selection leads to the divergence of LWS and consequently speciation of Lake Victoria rock cichlids. As described earlier, each Mwaburugu species has a unique LWS sequence. We investigated whether the differentiation was caused by natural selection. To detect such a signature of natural selection, we calculated the genetic differentiation among species through the 25-kb DNA fragments including LWS using sliding window analysis. As shown in figure 4A, the F_ST values for LWS regions highlighted in pink were higher in all the species pairs (fig. 4A; F_ST > 0.8) than those of upstream and downstream regions highlighted in blue (fig. 4A, F_ST < 0.5). The high F_ST value can be caused either by strong genetic differentiation between species by natural selection or by reduction of interspecific variations caused by reduction of polymorphisms within species due to genetic drift (Charlesworth 1998). In this case, the high K values (high genetic differentiation) for LWS region between

**FIG. 3.** Dark-light difference spectra of visual pigments. Visual pigments were reconstituted from (A) 3 LWS alleles (M3, Py, and Sp) with A1 and A2 retinal, (B) 2 RH1 alleles (104V and 104I) with A1 and A2 retinal, (C) 5 RH2Aβ alleles (β1–5) with A1 and A2 retinal, (D) RH2Aα with A1 and A2 retinal, (E) SWS2A with A1 and A2 retinal, and (F) SWS2B with A1 retinal. The λ_max values are indicated with their standard errors (nm) under each spectrum.
species were observed in all six species pairs (supplementary fig. S4A, Supplementary Material online), and the low π values (reduction of polymorphisms) for this region within species were not observed in all species except H. sp. “macula” (supplementary fig. S4A, Supplementary Material online). These results indicate that the LWS region may have been differentiated by divergent selection acted on this region. If divergent selection had acted on the LWS region, the polymorphism in the LWS region within species should be reduced in comparison with those in other loci. Indeed, a significant reduction of polymorphism within species compared with divergence between species was observed at the 25-kb region around the LWS gene in 2 species (table 2; McDonald test, H. sp. “macula”: P < 0.001; P. degeni: P < 0.001) and at the LWS region in H. sp. “macula” (table 2; MLHKA test, k < 1; a signature of reduction of polymorphism, k = 0.06, P < 0.001), indicating that divergent selection acted on this region. Tajima’s D indicates that LWS has a significant signature of selection in H. fischeri (table 2; Tajima’s D = –2.58, P < 0.001). The molecular signature of divergent selection found for LWS in H. sp. “macula” and H. fischeri suggests that the LWS region has diversified and is dominated by a single allele in each species, leading to the LWS diversity between species. In addition to these two species, although neutrality tests did not show the significant results after sequential Bonferroni correction, the low k value at LWS of H. pyrrhocephalus in MLHKA test (k = 0.16, P < 0.05) and the large negative value at LWS of P. degeni in Tajima’s D test (table 2; Tajima’s D = –1.98, P < 0.05) as well as the high FST values around LWS suggested that divergent selection also acted on LWS in H. pyrrhocephalus and P. degeni.

In rocky habitat cichlids, the adaptation of LWS alleles to different ambient light environments was reported (Terai et al. 2006; Seehausen et al. 2008). In contrast to the rocky shore, the sympatric species we used for our study inhabit the homogeneous ambient light environment. Accordingly, it is possible that the species-specific LWS-dependent color vision maintained by selection may adapt to light environments species-specifically recognized through their species-specific ecological traits, that is, feeding behaviors and avoidance of predators. What kinds of ecological traits are relevant to selection acting on LWS in the present case? Because the importance of vision in determining foraging efficiency has been well documented in Lake Victoria cichlids, it is possible that
they use their vision to search for food using the contrast between the food sources and the background (Fryer and Iles 1972; van der Meer and Bowmaker 1995). Although feeding behaviors of sympatric species in Mwaburugu were not observed, those of the same species inhabiting different localities were known to be diversified among species (H. sp. “macula” is Epilithic algae grazers [Seehausen 1996]; H. pyrrhocephalus is zooplanktivore, and H. fischeri and P. degeni are Molluscivore [Greenwood 1960]), indicating that feeding behaviors are one of candidate traits for selection acting on LWS in sympatric species in Mwaburugu.

Highly differentiated regions outside of LWS were also observed. The sliding window analysis for the 25-kb DNA fragments showed the high FST values in the 5-kb region upstream of LWS (fig. 4A). One possible explanation for this finding is that the hitchhiking effect on this upstream region by selection on LWS might increase the FST values for this region. Alternatively, this upstream region may be involved in cis-regulation for the expression of LWS that had been differentiated by selection. In zebrafish, the locus control region (LCR), located midway between SWS2 and LWS, regulates the LWS expression (Tsujimura et al. 2010), and a putative LCR in this region was suggested in cichlids (O’Quin et al. 2011) and livebearing fish (Watson et al. 2010; Tam et al. 2011). The high FST values that were observed between SWS2A and SWS2B (fig. 4A) might also be due to the putative LCR regulating SWS2A and/or LWS.

In the 7-kb DNA of RH1 and its flanking regions, the high FST values were observed in the RH1 coding region (fig. 4B). High differentiation of RH1 indicates divergent selection on this gene that was supported by the McDonald test in three species (table 2; H. sp. “macula”; P < 0.0001, H. pyrrhocephalus: P < 0.001, P. degeni: P < 0.001, H. fischeri: P < 0.11), but not by MLHKA and Tajima’s D tests owing to the low number of variable sites in RH1. Although MLHKA tests did not show significant results after sequential Bonferroni correction, the low k value at RH1 of H. sp. “macula,” H. pyrrhocephalus, and H. fischeri in MLHKA test (table 2; H. sp. “macula”; k = 0.18, P = 0.018; H. pyrrhocephalus: k = 0.14, P = 0.005; H. fischeri: k = 0.09, P = 0.008) as well as the high FST values around RH1 suggested that selection acted on RH1 in these Mwaburugu species. The θmax values, however, were the same between RH1 104V and 104I alleles (table 1). Besides their role in light absorption, the functional importance of substitutions in RH1 pigments in the reaction speed after photoreception, which is responsible for effective transduction of photic signals, has been reported (Sugawara et al. 2010). The amino acid at position 104 differentiating the 104V and 104I alleles may have a different function in the phototransduction process and may contribute to the adaptation of Lake Victoria cichlids. Future investigation of the LCRs of SWS2A, SWS2B, and LWS, as well as a possible function at the position 104 in cichlid RH1, may reveal their roles in visual adaptation.

Species-Specific Color Vision and Divergence of Male Nuptial Color May Maintain Species Diversity

As noted earlier, in rocky shore of Lake Victoria, pre-zygotic reproductive isolation through female mating preference to the nuptial coloration of conspecific male is thought to prevent gene flow among closely related cichlid species; for

Table 2. Neutrality Tests for Opsin Genes.

<table>
<thead>
<tr>
<th>Locus</th>
<th>P-value of McDonald Test</th>
<th>k</th>
<th>Tajima’s D</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ma</td>
<td>py</td>
<td>fi</td>
</tr>
<tr>
<td>LWS region</td>
<td>&lt;0.001*</td>
<td>0.16</td>
<td>0.08</td>
</tr>
<tr>
<td>SW52A</td>
<td>1.45</td>
<td>0.77</td>
<td>1.35</td>
</tr>
<tr>
<td>SW52B</td>
<td>0.73</td>
<td>0.17</td>
<td>0.61</td>
</tr>
<tr>
<td>LWS</td>
<td>0.05*</td>
<td>0.14</td>
<td>0.48</td>
</tr>
<tr>
<td>LWS-down</td>
<td>1.31</td>
<td>0.78</td>
<td>1.01</td>
</tr>
<tr>
<td>RH1 region</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>0.10</td>
</tr>
<tr>
<td>RH1-up</td>
<td>0.18</td>
<td>0.14*</td>
<td>0.09</td>
</tr>
<tr>
<td>RH1-down</td>
<td>1.43</td>
<td>0.42</td>
<td>1.18</td>
</tr>
<tr>
<td>Others</td>
<td>1.46</td>
<td>-1.50</td>
<td>-0.85</td>
</tr>
<tr>
<td>SW51</td>
<td>0.55</td>
<td>-0.89</td>
<td>0.62</td>
</tr>
<tr>
<td>RH2Aα</td>
<td>1.14</td>
<td>1.59</td>
<td>1.19</td>
</tr>
<tr>
<td>RH2B</td>
<td>-0.30</td>
<td>0.22</td>
<td>-0.14</td>
</tr>
</tbody>
</table>

Note.—Italicized values indicate statistically significant before sequential Bonferroni correction (P < 0.05). * indicate statistically significant after sequential Bonferroni correction (P < 0.006 for McDonald test; P < 0.001 for MLHKA and Tajima’s D test).

k value of MLHKA test.
Species are abbreviated as in figure 2.
The region downstream of LWS.
The region upstream of RH1.
The values were calculated from 4 kb including the RH1 and upstream and downstream regions.
The region downstream of RH1.
example, Pundamilia pundamili and P. nyerei (Seehausen et al. 1997, 2008; Seehausen and van Alphen 1998; Maan et al. 2006). Pundamilia pundamilia, whose males have bluish nuptial color, and P. nyerei, whose males have reddish nuptial color, are distributed in shallower (abundant in blue light) and deeper (abundant in red light) water, respectively, and each species adapts their LWS alleles to such ambient light environments (Seehausen et al. 2008). A female of P. nyerei, which has a red-shifted LWS allele, chooses a reddish conspecific male because that coloration pattern stimulates the female’s visual system more than those of other males. Also, a female of P. pundamilia, which has a blue-shifted LWS allele, chooses a bluish conspecific male more than other males (Seehausen et al. 2008). In the case of sympatric species in Mwaburugu, LWS allele-dependent diversity of color vision was suggested by the allele sequences and the absorption spectra (figs. 1 and 3; supplementary fig. S3, Supplementary Material online; and table 1), and reproductive isolation among species was significantly shown by mtDNA differentiation (fig. 2A). According to these results as well as the earlier-described female preference by colors that are tuned to the female’s visual system, we hypothesized that if the Mwaburugu species are also reproductively isolated through mate choice using color signals, the sensitivity of LWS pigments should correlate with the nuptial coloration of conspecific males in each species. To test this hypothesis, we analyzed the nuptial coloration of Mwaburugu species and calculated the correlation between the nuptial color and the light sensitivity of LWS pigments.

Light reflectance is generally applied to the measurement for animal color (e.g., bird color [Hill and McGraw 2006]), including cichlids (Maan et al. 2006; Dalton et al. 2010). Initially, we attempted to measure the light reflectance of live male cichlids of Lake Victoria, but we could not do it accurately because the body color of male cichlids changes during measurements. Accordingly, we wanted to substitute the measurement of light reflectance with another method, by which we could define the male nuptial color in digital photos with help of the hue index in the chart of MC, which is composed of 20 MHs. Any color seen by human vision can be categorized by one of the most similar MCs within one of the 20 MHs. Because it takes less time for us to take photographs than to measure the light reflectance of different areas on a male many times and hence the fish colorations in the photograph is nearer to natural coloration, it has advantage over the measuring of light reflectance. Therefore, we used the method of taking the photograph to evaluate the natural coloration of the males. Moreover by using photographs of cichlids, we could evaluate the fish colors by deducing the MC in wider areas of all body parts. This method is also advantageous because in the case of the measurement of the fish reflected light, it usually focused on the restricted and narrow area of the body. Using this MC, we can deduce the value of $\lambda_{\text{d}}$, which is represented by the monochromatic wavelength value.

MSP studies have reported that some Lake Victoria cichlid species had three different classes of cone photoreceptor for color vision, each of which absorbed different wavelength light: SWS2 for blue light, MWS for green, and LWS for red (van der Meer and Bowmaker 1995; Carleton et al. 2005). Analysis of the opsins gene expression of Lake Victoria cichlids supported the results of these works. Lake Victoria cichlids have seven cone opsins genes for color vision and mainly expressed SWS2A for blue light, RH2A (RH2Aor and/or RH2Aβ) for green, and LWS for red (Hofmann et al. 2009). Hence, they have trichromatic vision, consisting of blue, green, and red photoreceptors, and a relative lack of UV receptors. This photoreceptor composition is similar to that of humans. Therefore, considering that $\lambda_{\text{d}}$ can roughly represent the hue of the male nuptial color, we can postulate that the MC perceived by humans and its corresponding $\lambda_{\text{d}}$ value are likely to capture essential aspects of the coloration perceived by Lake Victoria cichlids as well as other fishes (Svensson et al. 2009).

To compare the male coloration between species, we defined the MCs in the five areas of lateral body (fig. 5A, areas numbered 1–5). We used 36 male individuals in total from six species. In each of these five areas, we identified the hue of the body color (MC) by the MH. In each species, the ratio of each MH class among the total MHs was plotted with reference to previous work (McElroy et al. 1991). As shown in figure 5B–G, the radar charts clearly indicated that the MH pattern was unique to each species (Fishers’s exact test using MH defined by M.A.: $\chi^2 = 185$, df = 90, $P < 0.001$, Fisher’s exact test using MH defined by S.M.: $\chi^2 = 218$, df = 90, $P < 0.001$). Platytaeniodus degeni, the most bluish species, showed a peak value at blue (fig. 5B, MH: 5B). H. ripianus and H. fischeri mainly showed a peak value at blue–green (fig. 5C and D, MH: 10BG) and additionally showed yellow (fig. 5C and D, MH: 10Y–5Y). H. sp. “thick skin” mainly showed a peak value at yellow–orange (fig. 5E, MH: 5Y–5YR) and blue (fig. 5E, MH: 10BG). Haplochromis pyrrhocephalus showed a peak value at yellow–orange (fig. 5F, MH: 5Y and 5YR). H. sp. “macula,” the most reddish species, mainly showed red and yellow (fig. 5G, MH: 10R–5R and 10Y). Moreover, such distinct coloration in each species correlated with the $\lambda_{\text{max}}$ value of LWS in each species. Namely, the LWS pigments reconstituted from the LWS allele (H allele in table 1) of reddish species (H. sp. “macula” in fig. 5G) absorbed longer wavelengths compared to those of bluish species. Also, the LWS pigments reconstituted from the LWS allele (Sp allele in table 1) of bluish species (P. degeni in fig. 5B) absorbed shorter wavelengths than those of reddish species.

Next, we statistically tested a correlation between the sensitivities of LWS pigments ($\lambda_{\text{max}}$) and the male nuptial color represented by the average $\lambda_{\text{d}}$ value for each species. A null hypothesis of the same $\lambda_{\text{d}}$ distributions in one species among several species was significantly rejected by multivariate analysis of variance (MANOVA) test (fig. 5H, MANOVA Wils’ $\lambda$, $P < 0.001$). We performed Spearman’s rank correlation test with several A1/A2 ratio (A2 ratios: 0%, 25%, 50%, 75%, and 100%). Because the rank orders of the $\lambda_{\text{max}}$ values estimated from each LWS allele were the same in each A1/A2 ratio, the different A1/A2 ratio provided the same results for the correlation in this test. Therefore, we showed only the results...
using the representative A1/A2 ratio (A1: A2 = 50% : 50%) for following tests. The correlation between the average $\lambda_d$ and $\lambda_{\text{max}}$ was not significant (fig. 6A, Spearman’s rank correlation coefficient: $Rs = 0.33$, $P > 0.23$). This is probably because the $\lambda_{\text{max}}$ value in $H. \text{pyrrhocephalus}$ is deviated from the correlation as shown in figure 6A. Presumably the property of opsin genes and the ratio of A1/A2 in $H. \text{pyrrhocephalus}$ is specific to this species resulting in a unique consequence of the color vision, which we describe in the later section. When $H. \text{pyrrhocephalus}$ was excluded, the correlation became stronger (fig. 6B, Spearman’s rank correlation coefficient: $Rs = 0.89$, $P = 0.041$), indicating the suggestive correlation between the $\lambda_{\text{max}}$ o fL W S and the average $\lambda_d$ of lateral body colors. This suggestive correlation matches well with our hypothesis that the reproductive isolation by mate choice through color signals is responsible for preventing interspecific hybridization.

One of the rationales to validate the exclusion of $H. \text{pyrrhocephalus}$ from the correlation analysis is the MSP data of this species from the population of Mwanza Gulf (fig. 1A),

**Fig. 5.** Male nuptial coloration variation and correlation of male nuptial color with $\lambda_{\text{max}}$ of LWS pigments. (A) The lateral cichlid body was divided into five areas by coloration pattern. These areas were used for measurement of male nuptial coloration using the MC chart. (B–G) Distribution of Munsell Hue (MH) of male lateral body (areas 1–5) in six Mwaburugu species. Color boxes outside of the radar chart indicate color images of MHs: clockwise from top, 5R, 10R, 5YR, 10YR, 5Y, 10Y, 5GY, 10GY, 5G, 10G, 5BG, 10BG, 5B, 10B, 5PB, 10PB, 5P, 10P, 5RP, and 10RP (see Materials and Methods section). For each MH class, filled circle is expressed as percentage of the total MHs collected. The scales on radial line indicate the percentage with respect to each 10% of the total MHs and center indicate 0%. Numbers in parentheses indicate the number of specimens. (H) Boxplot (median, quartiles and range) showing average $\lambda_d$ of male lateral body which were calculated based on MCs from five body areas (fig. 5A, areas 1–5) and two observers. Red lines in the box indicate the average $\lambda_d$. Species are abbreviated as in fig. 2.

**Fig. 6.** Correlation plots between average $\lambda_d$ of male lateral body and $\lambda_{\text{max}}$ of LWS pigments. Correlation plots between average $\lambda_d$ of male lateral body (areas 1–5) and $\lambda_{\text{max}}$ of LWS pigments in the case of (A) including $H. \text{pyrrhocephalus}$ and (B) excluding $H. \text{pyrrhocephalus}$. (A) Red arrow indicates that shift of $\lambda_{\text{max}}$ of $H. \text{pyrrhocephalus}$ (py) by high A2 usage. $\lambda_{\text{max}}$ of LWS pigments reconstructed from the dominant LWS allele groups (fig. 1D) were estimated in the case of an equal A1: A2 ratio. $Rs$, Spearman’s rank correlation coefficient. *$P < 0.05$, **$P > 0.05$. 

Visual Sensitivity Correlated with Nuptial Color. doi:10.1093/molbev/mss139 MBE
which suggested that this species uses A2 pigments more than other Lake Victoria cichlids (table 1, van der Meer and Bowmaker 1995; Carleton et al. 2005)). Three Haplochromis species coexisting with H. pyrrhocephalus as well as Pundamilia species showed the close $\lambda_{max}$ to one another in RH1 (Rods) (table 1). Haplochromis: 503.4–507.2 nm, Pundamilia: 503–506 nm) and LWS (table 1, Haplochromis: 565.0–569.3 nm, Pundamilia: 567–571 nm). These MSP works suggested that many Lake Victoria cichlid species used the A1/A2 pigments at the similar ratio (A2 pigment ratio in Pundamilia species: 34–58%) beyond genus. This notion is supported by the fact that Lake Victoria cichlids are very closely related genetically to one another beyond genus (see Introduction Section). Haplochromis pyrrhocephalus, however, showed the longer $\lambda_{max}$ value of MSP in Rods and LWS (table 1, Rods: 519.4 nm, LWS: 594.5 nm) than the other coexisting Haplochromis and Pundamilia species, suggesting that the A2 pigment usage of H. pyrrhocephalus was estimated to be higher than those of the other Lake Victoria cichlid species. In the case of H. pyrrhocephalus in Mwaburugu, if this assumption can be applied, the absorption spectra of LWS pigments became more congruent with the hypothesis that the Mwaburugu species are reproductively isolated through mate choice using color signals. Another rationale is that genes for SW5B and RH2Ax of H. pyrrhocephalus were subject to pseudogenization (table 3), whereas these genes in other Lake Victoria cichlids were reported to be expressed (Hofmann et al. 2009), suggesting that the usage of opsin genes in H. pyrrhocephalus has been specialized during their evolution.

Although H. sp. “thick skin,” H. riponianus, and H. fischeri have the same dominant LWS allele M3 (fig. 1E), these three species were genetically differentiated as significantly shown by mtDNA analysis (fig. 2A), indicating that the reproductive isolation is established and the gene flow is eliminated. Their colorations were different among these species (fig. 5C–F and 5H), and their color patterns (i.e., distribution of black bars, bright color on fin edge) were also differentiated. They may use the color pattern (i.e., distribution of black bars, bright color on fin edge) in addition to the lateral body coloration to recognize respective conspecific mates as well as other Lake Victoria cichlids (Seehausen 1996).

The Mwaburugu species are thought to be the secondary contact stage after their speciation and migration, because the eastern region of Speck Gulf including Mwaburugu is thought to have been frequently dried up in recent fluctuation of water level (Nicholson 1998). Interspecific mate choice using color signals may play a key role in preventing interspecific hybridization among sympatric species during the secondary contact and in maintaining sympatric species richness in Lake Victoria cichlids.

This study provides, for the first time, possible molecular and genetic evidence that visual diversity may maintain species richness in sympatric coexisting species. It is well known that several vertebrates might choose a mate based on species-specific color signals (i.e., male courtship signals and nuptial colors) to achieve sympatric species richness. The coloration on dewlaps of male Anolis lizards and the body coloration of Hamlet fishes are well-known examples of color signals that females recognize as features of their conspecific mating partners (Puebla et al. 2007; Losos 2009). The present work for the maintenance system of the sympatric species diversity in cichlids provides an important clue to elucidate the molecular bases of mate choice by color signals in the species exemplified above and how species richness can be sympatrically maintained in general.

### Supplementary Data

Supplementary figures S1–S4 and supplementary file S1 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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